Inhibition of the Chymotrypsin-like Activity of the Pituitary Multicatalytic Proteinase Complex†

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ABSTRACT: The multicatalytic proteinase complex (MPC), also referred to as proteasome, is a large molecular mass intracellular particle (~700 kDa), which exhibits three distinct proteolytic activities designated as chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH), all sensitive to inhibition by 3,4-dichloroisocoumarin (DCI). The presence of a component resistant to inhibition by DCI with an apparent preference toward bonds on the carboxyl side of branched-chain amino acids has also been recently established. Peptide aldehydes and peptide α-keto esters containing a hydrophobic residue in the P_1 position have been tested as potential inhibitors of the chymotrypsin-like activity. Three peptide aldehydes, (benzyloxycarbonyl)-Leu-Leu-phenylalaninal (Z-LLF-CHO), *N*-acetyl-Leu-Leu-norleucinal (Ac-LLnL-CHO), and *N*-acetyl-Leu-Leu-methioninal (Ac-LLM-CHO) were found to be slow-binding reversible inhibitors with K_i values of 0.46, 5.7, and 33 μM, respectively. The simplest kinetic model for inhibition is consistent with a mechanism involving a slow and reversible association of the enzyme with the inhibitor to form a EI complex. The aldehyde inhibitors also inhibited the trypsin-like and PGPH activities of the complex albeit with much higher K_i values than those for chymotrypsin-like activity. Z-LLF-CHO, the most selective of the three aldehydes, did not inhibit the PGPH activity at concentrations of up to 200 μM and inhibited the trypsin-like activity with a K_i ~2 orders of magnitude higher than that for the chymotrypsin-like activity. The activity of the DCI-resistant component was not affected by Z-LLF-CHO. Of the several peptide α-keto ester inhibitors tested, Z-Leu-Leu-Phe-COOEt was the most potent inhibitor of the chymotrypsin-like activity with a K_i of 53 μM. Although this K_i was 2 orders of magnitude higher than that for Z-LLF-CHO, this peptide α-keto ester was more specific, inhibiting exclusively the chymotrypsin-like activity. Unlike the aldehyde inhibitors the α-keto ester behaved as a classical competitive inhibitor. The usefulness of Z-LLF-CHO for identifying cleavages catalyzed by the chymotrypsin-like activity of the MPC in biologically active peptides is shown.

The multicatalytic proteinase complex (MPC),[1] an extralysosomal, predominantly cytoplasmic protease present in all eukaryotic cells, constitutes up to 1% of the protein in tissue homogenates [for reviews, see Orlowski (1990) and Rivett (1989a)]. The term multicatalytic has been proposed (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983) because of evidence showing that the complex can catalyze cleavage of peptide bonds on the carboxyl side of basic, acidic, and hydrophobic amino acid residues in both natural peptides and synthetic substrates, and because of evidence showing that each of these activities is associated with a distinct component of this multimeric complex. On the basis of the structure of the residue in the P_1 position of the substrate, the three activities have been designated as trypsin-like, peptidylglutamyl-peptide hydrolyzing, and chymotrypsin-like. However, unlike trypsin or chymotrypsin, the complex does not degrade simple synthetic substrates such as Z-Arg-2NA or Glt-Phe-2NA respectively. The mechanistic classification of the complex is still not conclusively established although evidence based on inhibitor studies with 3,4-dichloroisocoumarin (Harper et al., 1985) and other isocoumarin derivatives suggests that all components of the complex contain a serine residue in the active site (Orlowski & Michaud, 1989; Rivett, 1989b; Mason, 1990). In contrast to most serine proteinases, the complex is resistant to inhibition by DFP and PMSF, and although molecular cloning has provided the primary sequence of most of the subunits of the MPC, no homology has been found between these sequences and any of the known sequences of proteases (DeMartino et al., 1991; Emori et al., 1991; Fujiwara et al., 1989, 1990; Haass et al., 1989, 1990a,b; Kumatori, 1990; Tamura et al., 1990, 1991; Tanaka et al., 1990).

The MPC is composed of 13–15 nonidentical subunits with molecular weights ranging from 21 000 to 34 000 (Orlowski, 1990). The subunits are arranged in a stack of four rings, each containing six to eight subunits (Arrigo et al., 1988; Kopp et al., 1986). Although the presence of at least four distinct proteolytic activities each associated with a separate component of the complex has been well documented, the identity of the subunits expressing proteolytic activity is not

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[1] Abbreviations: Conventional one- and three-letter abbreviations are used for amino acids. Abu; α-aminobutyrate; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; Glt, glutaryl; HPLC, high-pressure liquid chromatography; LHRH, luteinizing hormone-releasing hormone; Me_2SO, dimethyl sulfoxide; MPC, multicatalytic proteinase complex; nL, norleucine; NMR, nuclear magnetic resonance; 2NA, 2-naphthylamide; OSu, *N*-hydroxysuccinimide ester; pAB, *p*-aminobenzoate; peptide-CHO, peptide aldehyde; peptide-COOEt, peptide ethyl α-keto ester; PGPHA, peptidylglutamyl-peptide hydrolyzing activity; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzoxycarbonyl.

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known and repeated attempts to isolate subunits with proteolytic activity have failed, leading to loss of activity. It is therefore likely that the structural integrity of the complex is necessary for expression of proteolytic activity. The allosterism of the complex is demonstrated by sigmoidal kinetics of the PGPHA (Orlowski et al., 1991) and observations that activation or inhibition of one activity of the complex can affect positively or negatively the activity of the other components (Wilk & Orlowski, 1983; Orlowski & Michaud, 1989; Dick et al., 1991).

Peptide aldehydes fulfilling the binding requirements of the substrate recognition site are known to be potent inhibitors of serine and cysteine proteases (Powers & Harper, 1986; Rich, 1986). Their tightness of binding had been proposed to derive from the formation of hemiacetals or thiohemiacetals and from the similarity of these enzyme–inhibitor adducts to the transition-state tetrahedral intermediates formed in reactions with substrates (Westerik & Wolfenden, 1972; Thompson, 1973).

We report here the synthesis of Z-Leu-Leu-phenylalaninal and data showing that this peptidyl aldehyde is a potent slow-binding inhibitor of the chymotrypsin-like activity of the MPC. Two structurally related inhibitors, Ac-LLnL-CHO (calpain inhibitor I) and Ac-LLM-CHO (calpain inhibitor II) also inhibit the same component by an apparently similar mechanism. Data are also presented showing that Z-LLF-COOEt is the best among several peptidyl α-keto esters inhibitors showing high selectivity toward the same component of the MPC.

MATERIALS AND METHODS

Materials. Bovine pituitaries were obtained from Pel Freeze Inc. (Rogers, AR). Z-Leu-Leu and LHRH were from Bachem Bioscience Inc. (Philadelphia, PA). 2,4-Dinitrophenylhydrazine, anhydrous Me_2SO, and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride were from Aldrich Chemical Co. (Milwaukee, WI). Ac-LLnL-CHO (calpain inhibitor I) and Ac-LLM-CHO (calpain inhibitor II) were obtained from Calbiochem (San Diego, CA). Silica gel HR extra pure was obtained from Brinkmann Instruments (Burlington, CA). All other reagents were obtained from Fisher Scientific Co. or from Sigma Chemical Co. (St. Louis, MO). Dichloroisocoumarin was synthesized as described previously (Harper et al., 1985). The synthesis of the peptide α-keto esters will be described separately.

Synthesis of Z-Leu-Leu-phenylalaninol. Phenylalaninol (756 mg, 5 mmol) was stirred overnight at 25 °C in 100 mL of tetrahydrofuran with 2.38 g of Z-LL-OSu (5.25 mmol) prepared by reacting Z-LL with *N*-hydroxysuccinimide (Anderson et al., 1964). The reaction mixture was filtered to remove a small amount of insoluble material and then evaporated to dryness in a rotary evaporator. The dried material was dissolved in 6 mL of methanol, and water was added dropwise until a light turbidity developed. The material was allowed to crystallize overnight, washed on a funnel with a small amount of 50% methanol, and dried. HPLC on a C_{18} reverse-phase column with a linear gradient between 10 and 60% acetonitrile containing 0.1% TFA for 20 min gave a single peak. The material was used in the next step without further purification.

Synthesis of Z-LLF-CHO. The peptide aldehyde was synthesized by oxidation of Z-Leu-Leu-phenylalaninol using the method of Pfitzner and Moffat (1965) essentially as modified by Wilk and Orlowski (1983). Z-Leu-Leu-phenylalaninol (511 mg, 1 mmol) was dissolved in 4 mL of anhydrous Me_2SO and 1.15 g of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (6 mmol) was added. After the solids had dissolved, 100 μL of 2 M anhydrous phosphoric acid in anhydrous Me_2SO was added. The formation of the aldehyde was monitored by testing the formation of the 2,4-dinitrophenylhydrazone after derivatization with 2,4-dinitrophenylhydrazine as described previously (Reingold & Orlowski, 1978). After completion of the oxidation reaction, the mixture was treated with 50 mL of water and the precipitated solid was collected and dried. The dried solid was dissolved in tetrahydrofuran, a small amount of insoluble material was removed by filtration, and the filtrate was evaporated to dryness under reduced pressure. The dried solid was applied to the top of a silica gel column in chloroform (3 cm × 52 cm) and then eluted with chloroform containing 1% methanol. Fractions of ~20 mL were collected, and their absorbance was monitored at 260 nm. The solvent in tubes showing the presence of material reacting with dinitrophenylhydrazine was pooled and evaporated to dryness. A white crystalline solid was obtained (yield, 84 mg): NMR (in $CDCl_3$) δ 0.8–1 [12 H, 4 d, $CH(CH_3)_2$], 1.4–1.8 [6 H, m, $CHCH_2CH(CH_3)_2$], 3.0–3.2 (2 H, m, $C_6H_5CH_2CH$), 4.2 (1 H, m, NHRC*H*CO), 4.4 (1 H, m, NHRC*H*CO), 4.6 (1 H, m, NHRC*H*CO), 5.1 (2 H, s, $C_6H_5CH_2OCO$), 5.2 (1 H, t, N*H*), 6.5 (1 H, d, N*H*), 6.8 (1 H, d, N*H*), 7.1–7.4 (10 H, m, $C_6H_5CH_2OCO$ and $C_6H_5CH_2CHCHO$), 9.58 (1 H, d, C*H*O); mp 73–75 °C. A single spot on silica TLC developed with 1% methanol in chloroform, R_f = 0.5.

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

Methods. (*a*) *Determination of Enzyme Activities with Synthetic Substrates.* The activities of the MPC were determined as described previously with substrate concentrations for experiments other than kinetic studies of 0.4 mM Cbz-D-Ala-Leu-Arg-2NA for the trypsin-like activity, 0.4 mM Cbz-Gly-Gly-Leu-pNA for the chymotrypsin-like activity, and 0.64 mM Cbz-Leu-Leu-Glu-2NA for the PGPHA (Wilk et al., 1979; Wilk & Orlowski, 1980, 1983).

(*b*) *Determination of Rates of Degradation of Natural Peptides.* The effect of Z-LLF-CHO on the rate of LHRH degradation was determined at 37 °C in reaction mixtures (final volume 50 μL) containing 50 mM Tris-HCl buffer (pH 8.0), 8 μM Z-LLF-CHO in Me_2SO, and the MPC (2.5 μg of protein). The final concentration of Me_2SO was 6.0%. After 1-h preincubation with inhibitor, the reactions were started by the addition of 12.5 nmol of LHRH. At time 0, 60, and 120 min, the reactions were terminated by addition of 10 μL of glacial acetic acid. Control reaction mixtures containing the same concentration of Me_2SO but no inhibitor were also carried through the procedure.

Rates of degradation of LHRH and formation of products were analyzed by subjecting the reaction mixtures to HPLC on a C_{18} μBondapak column (3.9 × 300 mm). Peptides were eluted from the column by a linear gradient established between 10 and 35% acetonitrile containing 0.1% TFA for 20 min. The amount of the peptide degraded was measured 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. The area under peaks of the products was also integrated, and the influence of inhibitor on the changes in the products was determined in relation to control incubation mixtures not containing the inhibitor.

The rate of degradation of dynorphin$^{1-8}$ was measured in reaction mixtures (final volume 50 μL) containing 50 mM Tris-HCl buffer (pH 8.0), 10 μg of the MPC, and 4 μM

Z-LLF-CHO in Me_2SO (final concentration 4%). After preincubation of the enzyme with the inhibitor for 1 h at 37 °C, the reaction was started by addition of 10 nmol of dynorphin$^{1-8}$. After 0, 30, and 60 min, the reactions were terminated by addition of 10 μL of glacial acetic acid. The rate of degradation was determined as described for LHRH using a linear gradient between 10 and 35% of acetonitrile in 0.1% TFA for 40 min.

Neurotensin$^{8-13}$ degradation was measured in incubation mixtures (final volume 115 μL) containing 50 mM Tris–HCl (pH 8.0 at 37 °C), 24 μg of DCI-treated MPC, 10 nmol of neurotensin$^{8-13}$, and up to 100 μM Z-LLF-CHO in Me_2SO (final concentration 1%). The DCI-treated enzyme was incubated with the aldehyde for 1 h at 37 °C in the assay buffer and the reactions were started by addition of neurotensin$^{8-13}$. After 90-min incubation the reactions were terminated by addition of 10 μL of glacial acetic acid. The products of degradation were separated on the μBondapak C_{18} column by eluting with a linear gradient between 10 and 35% acetonitrile in 0.1% TFA. The rate of product formation was analyzed as described above. Controls of the same composition but without inhibitor were also carried through the procedure.

(*c*) *Analysis of Amino Acid Composition of Peptide Products.* HPLC separation of peptides was carried out on a Waters 510 HPLC system equipped with a Shimadzu Chromatopac CR 601 data module. Amino acid analysis was carried out on a Rainin Rabbit HPLC system equipped with a Waters 745 data module and a Perkin-Elmer 650-15 fluorescence spectrophotometer. The amino acid composition of peptide products was determined after acid hydrolysis in evacuated tubes (6 N HCl at 110 °C for 24 h). Amino acids were analyzed fluorometrically after reaction with *o*-phthalaldehyde (Roth, 1971) as described previously (Chu & Orlowski, 1985).

(*d*) *Analysis of Slow-Binding Inhibition Kinetics.* Progress curves (Figure 1) for the inhibition of the chymotrypsin-like activity were obtained by incubating the MPC with 2 mM Z-Gly-Gly-Phe-pAB as the substrate in the presence of several concentrations of the aldehyde inhibitors (Z-LLF-CHO or Ac-LLnL-CHO). Incubations were carried out at 37 °C, in a circulating water bath. Reaction mixtures contained 50 mM Tris–HCl (pH 8.0), the MPC (60 μg), substrate, and several inhibitor concentrations in a final volume of 3.0 mL. At various times 250-μL aliquots of the mixture were withdrawn, and the reaction was terminated by addition of 100 μL of 25% TCA. The amount of free *p*-aminobenzoate released was determined after diazotization by sequential addition at 2-min intervals of 0.2 mL of 0.25% sodium nitrite, 0.2 mL of 1.25% ammonium sulfamate, and 0.4 mL of 0.125% naphthylethylenediamine dihydrochloride in 95% ethanol. The absorbance of the samples was measured at 555 nm, and the concentration of pAB was determined from a standard curve prepared under conditions of the experiment. The experimental data were fitted to eq 1 using the general curve fit feature of Kaleidagraph (a McIntosh data analysis/graphics application). Equation 1 describes the time course of product formation of an enzymatic reaction in the presence of a slow-binding inhibitor (Morrison, 1982), where v_s is the steady-state velocity, v_i is the initial velocity, k' is the pseudo-first-order rate constant which describes the rate of approach to the steady state of inhibition, and P is the amount of product formed at time t. v_s, v_i, and k' values for each inhibitor concentration were obtained.

$$P = v_s t + (v_i - v_s)[1 - \exp(-k't)]/k' \quad (1)$$

(*e*) *Determination of Initial K_i Values.* Initial K_i values for the chymotrypsin-like activity were measured without preincubation of the complex with the inhibitor. Short incubation times compatible with appropriate sensitivity of the assay (5 min) were used. Measurements were carried out in reaction mixtures containing 50 mM Tris–HCl, (pH 8.0 at 37 °C) at three substrate concentrations (0.5, 1, and 2 mM Z-GGF-pAB) and six different inhibitor concentrations. Substrates dissolved in Me_2SO were added to the incubation mixtures with the final concentration of Me_2SO not exceeding 4% by volume. Reactions were started by addition of 6–7.5 μg of MPC for the K_i determinations of the aldehyde inhibitors and 2.5 μg of MPC for the K_i determinations of all other inhibitors. After 5 min the reactions were terminated by addition of 100 μL of 25% TCA, and the amount of aromatic amine released was determined after diazotiazation as described above. K_i values were obtained from Dixon plots (1953) ($1/v$ versus [I]). The same procedure was used for the determination of initial K_i values for the trypsin-like activity at three substrate concentrations [0.1, 0.2, and 0.4 mM of Z-D-ALR-2NA] as the substrate.

(*f*) *Determination of Final K_i Values.* Final K_i values were determined under the same reaction conditions as the initial K_i with the following modifications. The enzyme (2.5 μg) was preincubated with various concentrations of the inhibitor for 60 min at 37 °C prior to the addition of the substrate to initiate the reaction. The reactions were allowed to proceed for 30 min and then terminated by addition of trichloroacetic acid.

(*g*) *Treatment of the Enzyme with 3,4-Dichloroisocoumarin (DCI).* MPC preparations (0.5 mg/mL) were incubated with DCI (final concentration 10 μM) for 60 min at 26 °C. The complex was then dialyzed for 24 h against a Tris–EDTA buffer (1 mM, pH 7.5). Under these conditions more than 90% of the chymotrypsin-like, trypsin-like, and PGPH activities were inactivated, whereas the activity of the DCI-resistant component measured with neurotensin as a substrate increased by a factor of 2–3 (Cardozo et al., 1992). The effect of Z-LLF-CHO on the activity of the DCI-resistant component of the complex was then determined using neurotensin$^{8-13}$.

RESULTS

Steady-State Kinetics of Inhibition of the Chymotrypsin-like Component. The inhibition of the chymotrypsin-like activity of the complex by aldehyde and α-keto ester analogs of substrates was determined without and with preincubation (initial K_i and final K_i, respectively) of the enzyme with the inhibitor. Preincubation of all three aldehyde inhibitors with the enzyme led to a significant decrease in K_i, indicating slow-binding kinetics (Table I). The ratio K_i(initial)/K_i(final) can be used as a measure of the slow-binding properties of the inhibitor (Kettner et al., 1988). A comparison of the K_i values for the aldehyde inhibitors shows that although their initial K_i values were similar the final K_i value for Z-LLF-CHO was 10 times lower than that for Ac-LLnL-CHO and more than 70 times lower than that for Ac-LLM-CHO. These differences suggest that an aromatic residue in the P_1 position increases the affinity of the inhibitor, a finding consistent with the designation of this component as chymotrypsin-like. Dixon plots (1953) produced lines intersecting near the y axis, making

Table I: Inhibition of the Chymotrypsin-like Activity of the Multicatalytic Proteinase Complex by Peptide Adehydes and Peptide α-Keto Esters[a]

inhibitor	[I] (μM)	initial K_i (μM)	[I] (μM)	final K_i (μM) (after preincub)
Z-LLF-CHO	12–60	22.5 ± 4.9 (15)	1.2–6.0	0.46 ± 0.14[b] (9)
Ac-LLnL-CHO	12–60	22.3 ± 1.3 (3)	2.4–12	5.7 ± 0.16[c] (3)
Ac-LLM-CHO	40–200	44.3 ± 8.4 (3)	20–100	33.4 ± 6.8[d] (3)
Z-LLF-COOEt	12–60	64 ± 3.5 (6)	12–60	53.2 ± 6.0 (6)
Z-LF-COOEt	60–300	220 ± 25 (3)	60–300	366 ± 46 (3)
Z-LnVCOOEt	120–600	1200 ± 6 (3)	120–600	1250 ± 175 (3)
Z-LLAbu-COOEt[e]	60–300	243 ± 9 (3)	60–300	238 ± 25 (3)

[a] Data are mean values ± SE. Values in parentheses represent the number of determinations. K_i values were determined by the method of Dixon (plot $1/v$ versus [I]) at six different inhibitor concentrations and at three different substrate concentrations (0.5, 1.0, and 2.0 mM). Activity was determined with Z-Gly-Gly-Phe-*p*-aminobenzoate as the substrate. Initial K_i values were obtained by determining activity after a 5-min incubation with the substrate without preincubation with inhibitor. Final K_i values were obtained by determining activity after a 30-min incubation with the substrate preceded by a 60-min incubation of the enzyme with the inhibitor. Both preincubations and incubations were at 37 °C for 60 min. [b] Significantly different ($p < 0.0001$) from values obtained without preincubation. [c] Significantly different ($p < 0.008$) from values obtained without preincubation. [d] Significantly different ($p < 0.03$) from values obtained without preincubation. [e] Abu, α-aminobutyrate.

Table II: Effect of Peptide Aldehydes and α-Keto Esters on Trypsin-like and Peptidylglutamyl-peptide Hydrolyzing Activities of the Complex[a]

inhibitor	activity	initial K_i (μM)	final K_i or IC_{50} (μM)
Z-LLF-CHO	trypsin-like	42.7 ± 3.8 (3)	42.7 ± 1.8 (3)
	PGPH	no inhibition	no inhibition
Ac-LLnL-CHO	trypsin-like	31.3 ± 0.5 (3)	50.5 ± 6.6 (3)
	PGPH	82	205
Ac-LLM-CHO	trypsin-like	86 ± 7.8 (3)	186 ± 6.6 (3)
	PGPH	200	280
Z-LLF-COOEt	trypsin-like	no inhibition	no inhibition
	PGPH	no inhibition	no inhibition

[a] K_i values were determined for the trypsin-like activity using the method of Dixon (1953) (plots $1/v$ versus [I]), at three different substrate concentrations and six inhibitor concentrations. IC_{50} values only could be determined for the PGPH activity because of the complicated sigmoidal relationship of the substrate concentration–velocity curves. Values in parentheses indicate the number of separate measurements. No inhibition of the PGPHA was observed with Z-LLF-CHO concentrations of up to 200 μM. No inhibition by Z-LLF-COOEt of the trypsin-like and PGPH activity was seen at concentrations of 200 and 300 μM, respectively. The difference between final and initial K_i values for the inhibition of the trypsin-like activity by the calpain inhibitor I was not statistically significant ($p = 0.088$).

it difficult to distinguish between competitive- and mixed-type inhibitions. Unlike Z-LLF-CHO, the corresponding alcohol, Z-LLF-OH, had no inhibitory effect at concentrations up to 300 μM.

The K_i values for the aldehyde inhibitors are markedly lower than those of the α-keto esters. The differences are especially dramatic when the final K_i values are compared. Thus, the final K_i value for the most potent of the aldehyde inhibitors (Z-LLF-CHO) was more than 2 orders of magnitude lower than that of the most potent α-keto ester (Z-LLF-COOEt). In contrast to the aldehyde inhibitors, the K_i values for the α-keto esters did not change after preincubation with the enzyme, suggesting that the association and dissociation of these inhibitors with the enzyme is a rapid equilibrium process. Plots of $1/v$ versus [I] gave lines intersecting close to x axis. Examination of the potency of inhibition of the α-keto esters as a function of structure shows that K_i values decreased when the number of amino acid residues in the inhibitor increased from two to three and that, as with the aldehyde inhibitors, an aromatic residue in the P_1 position is favored. A leucine residue in the P_3 position seems to contribute approximately 0.7 kcal/mol to the binding energy between the enzyme and the inhibitor (according to the relationship $\Delta G = -2.3RT \log K_1/K_2$, where K_1 and K_2 are K_i values for Z-LLF-COOEt and Z-LF-COOEt, respectively). Similar differences in the energy of binding were found between Z-LLF-COOEt and Z-LLAbu-COOEt, and between Z-LF-COOEt and Z-LnV-COOEt.

Effect of the α-Keto Ester and Aldehyde Inhibitors on the PGPH and Trypsin-like Activities of the Complex. It was important to determine whether the aldehyde and α-keto ester inhibitors of the chymotrypsin-like activity have any effect on the trypsin-like and PGPH activities of the complex. The results in Table II show no increase in binding for the trypsin-like and PGPH activities after preincubation with the inhibitors. The aldehyde inhibitors showed kinetics consistent with reversible competitive inhibition. The final K_i values for the trypsin-like activity were similar for both Z-LLF-CHO and Ac-LLnL-CHO, being some 100 times higher for the first and 10 times higher for the latter than the respective values for the chymotrypsin-like activity (see Table I). Ac-LLM-CHO was the weakest of the three aldehyde inhibitors in its effect on all three activities.

IC_{50} values (inhibitor concentration giving 50% inhibition) were determined for the PGPHA at a 0.64 mM substrate concentration and a range of inhibitor concentrations from 40 to 200 μM. Determination of K_i values for this component was not feasible because of its complex sigmoidal kinetics in plots of velocity versus substrate concentration. Whereas Z-LLF-CHO did not inhibit the PGPH activity at concentrations as high as 200 μM, Ac-LLnL-CHO inhibited this component with an IC_{50} of 80 μM. Preincubation of the enzyme with the inhibitor did not increase the IC_{50} values, indicating absence of slow-binding phenomena. Z-LLF-COOEt had no inhibitory effect on any of the two activities at concentrations as high as 300 μM, indicating that although this α-keto ester was less potent than the aldehyde inhibitors with respect to its effect on the chymotrypsin-like activity, it was nevertheless the most selective.

Kinetics of Inhibition of the Chymotrypsin-like Activity by the Aldehyde Inhibitors. As shown in Table I, the apparent affinity of the aldehyde inhibitors toward the chymotrypsin-like component of the complex increased when the enzyme was preincubated with these inhibitors prior to activity assays. Such behavior is consistent with slow-binding inhibition. In order to verify our final K_i values as well as to identify the mechanism by which inhibition is increased with time, a more detailed study of the kinetics of inhibition by aldehyde inhibitors was undertaken.

Three mechanisms which can account for slow-binding inhibition are (Morrison, 1982; Erion & Walsh, 1987)

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (A)$$

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \quad (k_2 \text{ slow}) \qquad (B)$$

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*I \quad (k_2 \text{ slow}) \qquad (C)$$

In mechanism A the formation of an enzyme–inhibitor complex occurs in a single but slow step. The low rate of binding in this mechnism could be due to either low inhibitor concentration or energetic barriers to binding. In mechanism B a relatively rapid binding of inhibitor to the enzyme is assumed followed by a slow conversion of the enzyme–inhibitor complex into a much tighter complex (EI*). The third mechanism C involves a slow conversion of the enzyme to form E*, which is the form of the enzyme capable of binding the inhibitor.

Experimentally, the slow-binding inhibition is evident from a lag in the inhibition observed in the progress curves of the reaction. The approach to the steady-state inhibition proceeds with the pseudo-first-order rate constant k'. The dependence of k' on inhibitor concentration, when the substrate concentration in the assay mixture is much lower than the K_m, can be described by eqs 2–4 for mechanisms A–C, respectively (Erion & Walsh, 1987; Bakker et al., 1990)

$$k' = k_2 + k_1[I] \qquad (2)$$

$$k' = k_4 + k_3[I]/([I] + K_i) \qquad (K_i = k_2/k_1) \qquad (3)$$

$$k' = k_1 + k_2K_i/([I] + K_i) \qquad (K_i = k_4/k_3) \qquad (4)$$

In mechanisms A and B k' should increase with increasing inhibitor concentrations, linearly for mechanism A and hyperbolically for mechanism B. For mechanism C the value of k' should decrease with increasing inhibitor concentration.

Figure 1 shows progress curves generated in the presence of four different concentrations of Z-LLF-CHO (1, 2, 3, and 4 μM). The experimental data were fitted to eq 1, as described in Methods, and the k', v_i, and v_s values were obtained for each inhibitor concentration. The initial velocity did not change with the change in the inhibitor concentration, as would be expected for mechanism A. A plot showing the dependence of k' on inhibitor concentration is shown in the inset of Figure 1. The values of k' increase linearly with increasing inhibitor concentration, which rules out mechanism C. Linear dependence of k' on the inhibitor concentration is consistent with the predictions of both mechanism A and mechanism B if the concentrations of the inhibitor used are well below the K_i. The apparent K_i for Z-LLF-CHO inhibition of the chymotrypsin-like activity is 23 μM (Table I). Thus, the concentrations used in the experiment are too low to discriminate between mechanisms A and B. Higher concentrations of the inhibitor (several times K_i) could not be used, since at such concentrations the approach to the steady state would be too rapid to measure by our methods. The reversibility of inhibition was shown by recovery of activity after dilution of the enzyme–inhibitor mixture into a substrate-containing assay solution.

Similar experiments were carried out with Ac-LLnL-CHO. The approach to steady-state inhibition with this inhibitor was much slower than with Z-LLF-CHO; therefore, higher

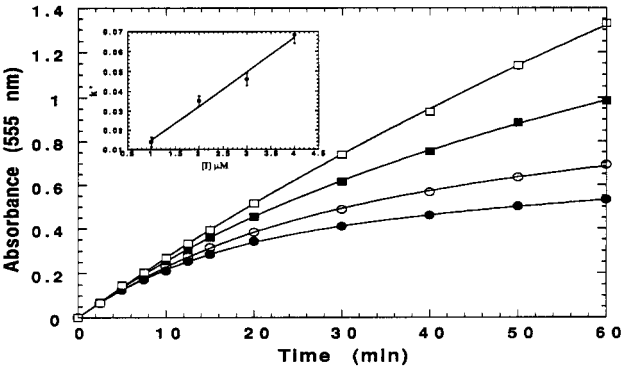

FIGURE 1: Progress curves of degradation of Z-GGF-pAB by the chymotrypsin-like activity in the presence of 1 (□), 2 (■), 3 (○), and 4 (●) μM Z-LLF-CHO. Activities were measured as described in Materials and Methods using 2 mM Z-GGF-pAB as the substrate. Lines were produced by computer fits of the experimental data points to eq 1. Regression coefficients of greater than 0.98 were obtained for each line. Inset: Dependence of k', the pseudo-first-order rate constant describing the rate of approach to the steady state of inhibition, on the concentration of Z-LLF-CHO. Values of k' were obtained from computer fits of progress curves of the chymotrypsin-like activity in the presence of variable concentrations of Z-LLF-CHO. Line values: k' intercept = –0.0029 and the correlation coefficient was equal to 0.99.

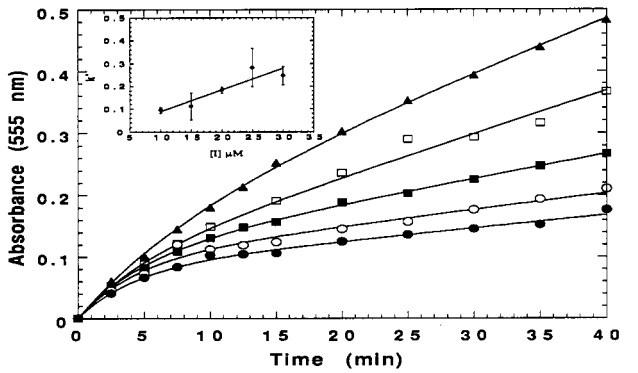

FIGURE 2: Progress curves of degradation of Z-GGF-pAB by the chymotrypsin-like activity in the presence of 10 (▲), 15 (□), 20 (■), 25 (○), and 30 (●) μM calpain inhibitor I (Ac-LLnL-CHO). Inset: Dependence of k', the pseudo-first-order rate constant describing the rate of approach to the steady state of inhibition, on the concentration of Ac-LLnL-CHO. Generation of lines and experimental conditions were the same as described in the legend to Figure 1. Line values: k' intercept = –0.006 and the correlation coefficient was equal to 0.92.

inhibitor concentrations (10–30 μM) were used to study the kinetics of binding. Values for k', v_s, and v_i were obtained from the computer fits to the experimental progress curves shown in Figure 2. Concentrations much above K_i could not be used because of the same limitations found in experiments with Z-LLF-CHO, and a plot of k' versus inhibitor concentration was linear (inset in Figure 2). The v_i value did not change with changes in inhibitor concentration and was the same for calpain inhibitor I and for Z-LLF-CHO. Thus, as with Z-LLF-CHO, inhibition of the MPC by Ac-LLnL-CHO is adequately represented by mechanism A. Values for k_{off} (k_2) calculated from the relationship $k_{off} = k'v_s/v_i$ (Morrison, 1982) were for Z-LLF-CHO and calpain inhibitor I $1.38 \times 10^{-4} \pm 3 \times 10^{-5}$ and $5 \times 10^{-4} \pm 4 \times 10^{-5}$ s^{-1}, respectively. The k_{on} (k_1) values calculated from the plots of k' versus inhibitor concentration (insets in Figures 1 and 2) were 283 and 158 M^{-1} s^{-1} for Z-LLF-CHO and calpain inhibitor I, respectively. From the k_{off} and k_{on} values it can be calculated that at least 75% of the steady-state rate was achieved at the lowest Z-LLF-CHO concentration (1 μM), whereas at the higher inhibitor

Table III: Effect of Aldehyde Inhibitors on Degradation of Natural Peptides[a]

peptide and sequence		products of degradation	inhibitor	inhib (%)
LHRH	pEHWSYGLRPG-NH_2	GLRPG-NH_2	Z-LLF-CHO	83 ± 9
	↑ ↑	pEHW	Z-LLF-CHO	54 ± 15
		pEHWSY	Z-LLF-CHO	86 ± 3
dynorphin$^{1-8}$	YGGFLRRI	YGGFL	Z-LLF-CHO	75 ± 5
	↑ ↑ ↑	YGGFLRR	leupeptin	83 ± 3
		YGGFLR	leupeptin	90 ± 1
neurotensin$^{8-13}$	RRPYIL	RRPYI	Z-LLF-CHO	0
	↑			

[a] Activities and the effect of inhibitors were determined as described in Materials and Methods. Degradation of neurotensin$^{8-13}$ was determined with the DCI-treated enzyme. Arrows indicate the site of cleavage. For other details, see the text.

concentrations of Z-LFF-CHO and at all concentrations of calpain inhibitor I virtually steady-state rates were achieved. K_i calculations from the values of k_{off}/k_{on} were 0.49 μM for Z-LLF-CHO and 3 μM for calpain inhibitor I, in excellent agreement with the final K_i values shown in Table I.

While mechanism A is the simplest model that can be applied for the analysis of the progress curves, the possibility that the reaction follows mechanism B cannot be excluded, because of methodological limitations in the analysis of initial velocities in the presence of high inhibitor concentrations. Analysis of the data according to mechanism B provides values for k_4 that are the same as those for k_{off} in mechanism A. Values of k_3 calculated as the product of the slope of k' versus inhibitor concentration and initial K_i are 6.3×10^{-3} and 3.5×10^{-3} s^{-1} for Z-LLF-CHO and Ac-LLnL-CHO, respectively. These values can be used for the calculation of k_4 from the relationship $k_3/k_4 = K_i/K_i^* - 1$, where K_i is the initial K_i and K_i^* is the final K_i (Morrison, 1982). Values of k_4 obtained in this manner are 1.4×10^{-4} and 1.18×10^{-3} s^{-1} for Z-LLF-CHO and Ac-LLnL-CHO, respectively, in good agreement with the k_4 values calculated by the first method.

Effect of Z-LLF-CHO on Peptide Degradation. The effect of Z-LLF-CHO on the degradation of natural peptides by the complex was studied using LHRH, dynorphin$^{1-8}$, and neurotensin fragment$^{8-13}$ as the substrates. Table III shows the sequences of these peptides as well as the sequences of the major degradation products formed by incubation with the MPC. LHRH degradation was inhibited by more than 80% in the presence of 8 μM Z-LLF-CHO. The amino acid composition of the products indicates that cleavages occurred on the carboxyl side of Trp and Tyr residues. The extent of inhibition of both these cleavages by Z-LLF-CHO suggests that they are catalyzed by the chymotrypsin-like activity of the complex, although cleavage after Tyr residues was inhibited to a greater extent than that after Trp.

The degradation of dynorphin$^{1-8}$ was studied in the presence and absence of 4 μM Z-LLF-CHO and in the presence and absence of 15 μM leupeptin. The amino acid composition of the degradation products indicates cleavages on the carboxyl side of both arginine residues and cleavage on the carboxyl side of the Leu residue with the formation of leucine enkephalin (YGGFL). Both cleavages after the arginyl residues were inhibited by leupeptin but not by Z-LLF-CHO. This indicates that these cleavages are catalyzed by the trypsin-like activity of the complex, since leupeptin was shown to specifically inhibit the activity of this component. Cleavage after the leucine residue was specifically inhibited by Z-LLF-CHO, but not by leupeptin, indicating that this aldehyde can at low concentrations specifically inhibit the chymotrypsin-like activity of the complex.

Work in this laboratory has shown that a DCI-resistant component of the complex cleaves specifically the Ile–Leu bond in neurotensin and in neurotensin fragment$^{8-13}$. Incubation of the DCI-treated enzyme with 10 μM Z-LLF-CHO showed that the rate of cleavage of this bond is not affected by the presence of the inhibitor, indicating that the DCI-resistant component is not sensitive to inhibition by the aldehyde.

DISCUSSION

Evidence based on specificity studies with synthetic and natural peptides and the effect of various inhibitors led to the conclusion that the MPC, a multimeric, high molecular weight predominantly cytoplasmic protease exhibits at least three distinct proteolytic activities. The three activities catalyze cleavage of peptide bonds on the carboxyl side of hydrophobic (chymotrypsin-like), acidic (PGPH), and basic (trypsin-like) amino acid residues, and each of the activities seems to be associated with a different component of the complex [for a review, see Orlowski (1990)]. Initial work with aldehyde inhibitors showed that the trypsin-like activity is specifically inhibited in a competitive manner by leupeptin ($K_i = 1.2 \times 10^{-6}$ M) and that the chymotrypsin-like activity measured with Z-Gly-Gly-Leu-pNA as substrate is competitively inhibited ($K_i = 2.5 \times 10^{-4}$ M) by Z-Gly-Gly-leucinal (Wilk & Orlowski, 1980, 1983; Orlowski & Wilk, 1981). More recent experiments (Orlowski & Michaud, 1989) have shown that all three activities are irreversibly inactivated by 3,4-dichloroisocoumarin, a general serine proteinase inhibitor, albeit with different pseudo-first-order rate constants. Treatment of the complex with DCI led also to the finding of a DCI-resistant component of the complex that showed a preference toward bonds on the carboxyl side of branched-chain amino acids in natural peptides and proteins (Cardozo et al., 1992) but was inactive toward Z-Gly-Gly-Leu-pNA and Z-Gly-Gly-Phe-pAB, two chromogenic substrates used for following the chymotrypsin-like activity. This DCI-resistant activity could be related to the caseinolytic activity described by Yu et al. (1991) and Pereira et al. (1992).

The presence of two distinct components cleaving bonds on the carboxyl side of hydrophobic residues and the need for assignment of cleavages in natural peptides and proteins to specific components of the complex made it necessary to search for inhibitors that could be used for this purpose. Furthermore, the availability of such inhibitors could be expected to facilitate studies on the function of the complex in intact cells and tissues. We therefore directed our attention toward peptidyl aldehydes and peptidyl α-keto esters with hydrophobic residues in the P_1 position as potential inhibitors.

Of the three peptidyl aldehydes tested, the Z-LLF-CHO synthesized in the course of this study was the most potent (final K_i = 0.46 μM), showing a marked selectivity toward the chymotrypsin-like component. Comparison of this inhibitor with the other two analogous aldehyde inhibitors (Ac-LLnL-CHO and Ac-LLM-CHO) shows that the aromatic phenylalaninal residue markedly contributes to inhibitor binding, a finding consistent with the specificity of this component. It is of interest that the same inhibitor had no effect on the DCI-resistant component even though its specificity is similar to the chymotrypsin-like component, albeit with a preference toward bonds on the carboxyl side of branched-chain amino acids. The basis for this selectivity is not clear. Since the inhibitory effect of peptidyl aldehydes is directed toward either serine or cysteine proteinases one could speculate that the DCI-resistant component is mech-

anistically distinct from these two classes of enzymes. It is however more likely that the lack of inhibition by the peptidyl aldehydes is simply the result of poor binding of the inhibitor, since unlike the chymotrypsin-like activity, which readily attacks small synthetic substrates, the DCI-resistant component does not cleave short synthetic substrates containing an aromatic amine as a chromogenic group and shows a marked preference toward longer natural peptides and proteins. Whatever the real basis of the selectivity of the Z-LLF-CHO inhibitor might be, our results show that this inhibitor can be used to distinguish between those bonds in natural peptides and proteins that are cleaved by the chymotrypsin-like and the DCI-resistant components of the complex.

It is notable that whereas all three aldehydes showed slow-binding kinetics, all the α-keto esters reacted as competitive rapid equilibrating inhibitors. Peptides containing aldehyde and α-keto ester functional groups are known to react with serine and cysteine proteinases to form hemiacetals or thiohemiacetals, respectively, or the corresponding ketals or thioketals in the case of α-keto esters (Lewis & Wolfenden, 1977; Chen et al., 1979; Brayer et al., 1979). These enzyme–inhibitor adducts are thought to resemble the tetrahedral intermediate formed during the catalysis of peptide bond hydrolysis (Westerik & Wolfenden, 1972; Thompson, 1973). Since both classes of inhibitors are capable of forming tetrahedral enzyme–inhibitor adducts, the reason for the difference in the kinetics of inhibition is not evident. The presence of an aldehyde group is necessary for slow binding since the corresponding alcohol (Z-LLF-OH) had no inhibitory effect. The presence, however, of adjacent hydrophobic residues must also contribute to slow binding since Z-Gly-Gly-leucinal in which the leucine residues were replaced by glycine residues showed no slow-binding kinetics (data not shown). The finding of slow-binding kinetics with the aldehyde but not keto ester inhibitors could also result from the binding of the aldehydes to multiple allosterically interacting sites, a possibility that needs to be considered in view of the potential of multiple binding sites in the complex.

Our studies of the kinetics of inhibition of the MPC by peptidyl aldehydes suggest that the inhibition occurs in a single unusually slow step. Such a slow-binding step could be due to a very low effective inhibitor concentration, to a slow change of the inhibitor structure which enables binding to the enzyme, and finally to a slow rearrangement of the enzyme–inhibitor complex which occurs without any kinetically distinguishable intermediates.

Aldehydes are known to exist in aqueous solutions in a hydrated and dehydrated form. The ratio of the two forms for Bz-F-CHO is 9:1 (Kennedy & Shultz, 1979). Since the hydrated form does not bind to the active site, the effective inhibitor concentration of the aldehyde constitutes only a fraction of the total aldehyde concentration. Assuming a value for the lower limit of the diffusion-limited "on" rate constant as 10^9 M^{-1} s^{-1} (Fersht, 1986), the k' value at an inhibitor concentration of 4 μM should be equal to 400 s^{-1} if 10% of the inhibitor is in the aldehyde form. This value is much higher than the 0.0001 s^{-1} experimental value of k' at 4 μM. Accordingly, low effective inhibitor concentration could not account for the slow binding observed with the aldehyde inhibitors.

The rate of the dehydration of *N*-benzoyl-L-phenylalaninal was determined to have a first-order rate constant of 0.3 s^{-1} (Kennedy & Schultz, 1979). The rate of dehydration of hydrated aldehyde is clearly too fast to account for the slow binding observed in our experiments.

In studies of transition-state analog inhibitors of thermolysin (Bartlett & Marlowe, 1987) and renin (Kati et al., 1987), a rate-limiting displacement of a water molecule at the active site was proposed to be responsible for slow binding. It is possible that a similar mechanism is the basis for the slow inhibition observed with the aldehyde inhibitors of the chymotrypsin-like activity.

Although mechanism A seems to be the simplest mechanism to account for our data, the two-step mechanism cannot be ruled out completely. The hyperbolic dependence of k' on the inhibitor concentration can be missed unless the concentration of the inhibitor used in the analysis of inhibition kinetics is much higher than the K_i for the initial binding step (Morrison, 1982). The apparent initial values of K_i measured for Z-LLF-CHO and Ac-LLnL-CHO of 22 μM are higher or approximately equal to the concentrations of these inhibitors at which analysis of slow-binding inhibition could be carried out accurately. Thus, we could not determine whether the k' value reaches saturation at higher inhibitor concentrations. Therefore, inhibition could occur by forming a loose enzyme–inhibitor complex with a K_i below the apparent initial K_i, followed by a change in the structure of the enzyme–inhibitor complex. The overall K_i for the second complex is then equal to K_i(final); $K_i(\text{final}) = [k_4/(k_3 + k_4)]K_i(\text{initial})$. The nature of the change in the enzyme–inhibitor complex is not clear. A comparison of X-ray structures of complexes of slow-binding and classical inhibitors with various proteases (Brady & Abeles, 1990; Brady et al., 1990; Bone et al., 1987; Delbaere & Brayer, 1985; Takahashi et al., 1988) failed to show a conformational change in the enzyme which accompanied slow-binding inhibition. As for mechanism A, a rate-limiting water molecule displacement could account for the slow-binding inhibition by the two-step mechanism.

Experiments with LHRH, dynorphin$^{1-8}$, and neurotensin$^{8-13}$ show the usefulness of the aldehyde inhibitor for identification of a component of the complex catalyzing cleavage of a specific peptide bond in a given amino acid sequence. In the case of LHRH, cleavage at the Tyr^5–Gly^6 and Trp^3–Ser^4 bonds was greatly inhibited at concentrations of the inhibitor that would be expected to affect primarily the chymotrypsin-like activity, suggesting involvement of this component of the complex. The observation that cleavage of the Trp^3–Ser^4 bond was inhibited to a lesser extent than cleavage of the Tyr^5–Gly^6 bond could have been the result of a higher affinity toward the enzyme (lower apparent K_m) of the sequence around the Trp residue.

The selective inhibition by Z-LLF-CHO of cleavage of the Leu^5–Arg^6 bond in dynorphin$^{1-8}$ shows that involvement of the chymotrypsin-like activity in peptide hydrolysis can be distinguished from the involvement of the trypsin-like activity. This was confirmed by showing that cleavage after the two arginine residues in dynorphin$^{1-8}$ was selectively inhibited by leupeptin, but not by Z-LLF-CHO. It should be noted that the DCI-resistant component with a specificity similar to that of the chymotrypsin-like activity (cleavage after branched amino acids) was not affected by Z-LLF-CHO. As already discussed, this could result from a preference of this component toward longer peptide structures than those presented by the short synthetic substrates. Although this component was not inhibited by 3,4-dichloroisocoumarin, it was nevertheless susceptible to inhibition by other modified isocoumarin derivatives such as 7-amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin (Kam et al., 1988), suggesting that its basic mechanism is the same as that of the other component of the complex. It is therefore possible that synthesis of peptidyl

aldehydes having a longer peptide structure better conforming with the binding requirements at this site could result in an effective inhibitor of this component.

REFERENCES

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839–1842.

Arrigo, A.-P., Tanaka, K., Goldberg, A. L., & Welch, W. J. (1988) *Nature 331*, 192–194.

Bakker, A. V., Jung, S., Spencer, R. W., Vinick, F. J., & Faraci, W. S. (1990) *Biochem. J. 271*, 559–562.

Bartlett, P. A., & Marlowe, C. K. (1987) *Biochemistry 26*, 8553–8561.

Bone, R., Shenvi, A. B., Kettner, C. A., & Agard, D. (1987) *Biochemistry 26*, 7609–7614.

Brady, K., & Abeles, R. H. (1990) *Biochemistry 29*, 7608–7617.

Brady, K., Wei, A., Ringe, D., & Abeles, R. H. (1990) *Biochemistry 29*, 7600–7607.

Brayer, G. D., Delbaere, L. T. J., James, M. N. G., Bauer, C. A., & Thompson, R. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 96–100.

Cardozo, C., Vinitsky, A., Hidalgo, M.-C., Michaud, C., & Orlowski, M. (1992) *Biochemistry 31*, 7373–7380.

Chen, R., Gorenstein, P. G., Kennedy, W. P., Lowe, G., Nurse, D., & Schultz, R. M. (1979) *Biochemistry 18*, 921–926.

Chu, T. G., & Orlowski, M. (1985) *Endocrinology 116*, 1418–1425.

Delbaere, L. T. J., & Brayer, G. D. (1985) *J. Mol. Biol. 183*, 89–101.

DeMartino, G. N., Orth, K., McCullogh, M. L., Lee, L. W., Munn, T. Z., Moomaw, C. R., Dawson, P. A., & Slaughter, C. A. (1991) *Biochim. Biophys. Acta 1079*, 29–38.

Dick, L. R., Moomaw, C. R., DeMartino, G. N., & Slaughter, C. A. (1991) *Biochemistry 30*, 2725–2734.

Dixon, M. (1953) *Biochem. J. 55*, 170–171.

Emori, Y., Tsukahara, T., Kawasaki, H., Ishiura, S., Sugita, H., & Suzuki, K. (1991) *Mol. Cell. Biol. 11*, 344–353.

Erion, M. D., & Walsh, C. T. (1987) *Biochemistry 26*, 3417–3425.

Fersht, A. (1986) in *Enzyme Structure and Mechanism*, 2nd ed., pp 303–304, 147, W. H. Freeman & Co., New York.

Fujiwara, T., Tanaka, K., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanaga, S., Kakizuka, A., & Nakanishi, S. (1989) *Biochemistry 28*, 7332–7340.

Fujiwara, T., Tanaka, K., Orino, E., Yoshimura, T., Kumatrori, A., Tamura, T., Chung, C. H., Nakai, T., Yamaguchi, K., Shin, S., Kakizuka, A., Nakanishi, S., & Ichihara, A. (1990) *J. Biol. Chem. 265*, 16604–16613.

Haass, C., Presold-Hurt, B., Multhaup, G., Beyreuther, K., & Kloetzel, P.-M. (1989) *EMBO J. 8*, 2373–2379.

Haass, C., Pesold-Hurt, B., & Kloetzel, P.-M. (1990a) *Nucleic Acids Res. 18*, 4018.

Haass, C., Pesold-Hurt, B., Multhaup, G., Beyreuther, K., & Kloetzel, P.-M. (1990b) *Gene, 90*, 235–241.

Harper, J. W., Hemmi, K., & Powers, J. C. (1985) *Biochemistry 24*, 7200–7213.

Hori, H., Yasutake, A., Minematsu, Y., & Powers, J. C. (1985) in *Peptides: Synthesis–Structure–Function*, Proceedings of the Ninth American Peptide Symposium (Deber, C. M., Hruby, J., & Kopple, K. D. Eds.) pp 819–822, Pierce Chemical Co., Rockford, IL.

Kam, C.-M., Fujikawa, K., & Powers, J. C. (1988) *Biochemistry 27*, 2547–2557.

Kati, W. M., Pals, D. T., & Thaisrivongs, S. (1987) *Biochemistry 26*, 7621–7626.

Kennedy, W. P., & Schultz, R. M. (1979) *Biochemistry 18*, 349–356.

Kettner, C. A., Bone, R., Agard, D. A., & Bachovchin, W. W. (1988) *Biochemistry 27*, 7682–7688.

Kopp, F., Steiner, R., Dahlmann, B., Kuehn, L., & Reinauer, H. (1986) *Biochim. Biophys. Acta 872*, 253–260.

Kumatori, A., Tanaka, K., Tamura, T., Fujiwara, T., Ichihara, A., Tokunaga, F., Onikura, A., & Iwanaga, S. (1990) *FEBS Lett. 264*, 279–282.

Lewis, C. A., & Wolfenden, R. (1977) *Biochemistry 16*, 4890–4895.

Mason, R. W. (1990) *Biochem. J. 265*, 479–484.

Morrison, J. F. (1982) *Trends Biochem. Sci. 7*, 102–105.

Orlowski, M. (1990) *Biochemistry 29*, 10289–10297.

Orlowski, M., & Wilk, S. (1981) *Biochem. Biophys. Res. Commun. 101*, 814–822.

Orlowski, M., & Michaud, C. (1989) *Biochemistry 28*, 9270–9278.

Orlowski, M., Cardozo, C., Hidalgo, M. C., & Michaud, C. (1991) *Biochemistry 30*, 5999–6005.

Pereira, M. E., Nguyen, T., Wagner, B. J., Margolis, J. W., Yu, B., & Wilk, S. (1992) *J. Biol. Chem. 267*, 7949–7955.

Pfitzner, K. E., & Moffat, J. G. (1965) *J. Am. Chem. Soc. 87*, 5661–5670.

Powers, J. C., & Harper, J. W. (1986) in *Proteinase Inhibitors* (Barrett, A. J., & Salvensen, G., Eds.) Research Monographs in Cell and Tissue Physiology, Vol. 12, pp 55–152, Elsevier Science Publishers, Amsterdam.

Reingold, D. F., & Orlowski, M. (1978) *J. Neurochem. 32*, 907–913.

Rich, D. H. (1986) in *Proteinase Inhibitors* (Barrett, A. J., & Salvensen, G. Eds.) Research Monographs in Cell and Tissue Physiology, Vol. 12, pp 153–178, Elsevier Science Publishers, Amsterdam.

Rivett, J. A. (1989a) *Arch. Biochem. Biophys. 268*, 1–8.

Rivett, J. A. (1989b) *J. Biol. Chem. 264*, 12215–12219.

Roth, M. (1971) *Anal. Chem. 43*, 880–882.

Takahashi, L. H., Radhakrishnan, R., Rosenfield, R. E., Jr., Meyer, E. F., Jr., Trainor, D. A., & Stein, M. (1988) *J. Mol. Biol. 201*, 423–428.

Tamura, T., Tanaka, K., Kumatori, A., Yamada, F., Tsurumi, C., Fujiwara, T., Ichihara, A., Tokunaga, F., Aruga, R., & Iwanaga, S. (1990) *FEBS Lett. 262*, 91–94.

Tamura, T., Lee, D. H., Osaka, F., Fujiwara, T., Shin, S., Chung, C. H., Tanaka, K., & Ichihara, A. (1991) *Biochim. Biophys. Acta 1089*, 95–102.

Tanaka, K., Fujiwara, T., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanaga, S., Kakizuka, A., & Nakanishi, S. (1990) *Biochemistry 29*, 3777–3785.

Thompson, R. C. (1973) *Biochemistry 12*, 47–51.

Westerik, J. O., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 8195–8197.

Yu, B., Pereira, M. E., & Wilk, S. (1991) *J. Biol. Chem. 266*, 17396–17400.

Wilk, S., & Orlowski, M. (1980) *J. Neurochem. 35*, 1172–1182.

Wilk, S., & Orlowski, M. (1983) *J. Neurochem. 40*, 842–849.

Wilk, S., Pearce, S., & Orlowski, M. (1979) *Life Sci. 24*, 457–464.

Williams, J. W., Morrison, J. F., & Duggleby, R. G. (1979) *Biochemistry 18*, 2567–2573.