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Peptidylglutamyl–Peptide Hydrolase Activity of the Multicatalytic Proteinase Complex: Evidence for a New High-Affinity Site, Analysis of Cooperative Kinetics, and the Effect of Manganese Ions†

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ABSTRACT: The multicatalytic proteinase (MCP) complex or proteasome is a major nonlysosomal proteinase of eukaryotic cells. The proteinase can cleave peptide bonds on the carboxyl side of hydrophobic, basic, or acidic amino acid residues. These activities have been referred to as "chymotrypsin-like", "trypsin-like", and "peptidylglutamyl–peptide hydrolase" activities, respectively, and have been shown to be catalyzed at distinct sites. The latter activity is often assayed with the synthetic peptide substrate Z-Leu-Leu-Glu-β-naphthylamide (LLE-NA). *N*-tBoc-Ala-Ala-Asp-SBzl is also a substrate for the rat liver MCP, suggesting a broader specificity for cleavage on the carboxyl side of acidic residues than the peptidylglutamyl–peptide hydrolase activity previously reported. The pH optimum is in the range of pH 7.0–7.5. Studies of the dependence of velocity on LLE-NA concentration show (a) that there is a high-affinity site (LLE1) which obeys Michaelis–Menten kinetics with a K_m value of ~100 μM and (b) that at higher substrate concentrations (LLE2) the curve is sigmoidal, suggesting either allosteric activation of the proteinase at a second site or the involvement of multiple catalytic sites which display positive cooperativity. Activity at the high-affinity site (LLE1) can be distinguished from that of the activity of the LLE2 component by the effect of inhibitors, divalent metal ions, and KCl, as well as by its response to heat treatment. The addition of 1 mM $MnCl_2$ stimulates both LLE1 and LLE2 activities and also permits saturation of MCP with substrate at concentrations of LLE-NA below the solubility limit of this peptide. Under these conditions, the Hill coefficient calculated for LLE2 is 5.1 and the $K_{0.5}$ value is 0.28 mM. Such activation of the MCP complex at high substrate concentrations should be advantageous for the regulation of proteinase activity within cells and for the rapid degradation of protein substrates.

The multicatalytic proteinase (MCP),[1] prosome, or proteasome is a complex multisubunit proteinase which is found in the nucleus and cytoplasm of eukaryotic cells [see Rivett (1989a) and Orlowski (1990) for reviews]. It appears to be involved in ubiquitin-dependent as well as ubiquitin-independent nonlysosomal pathways of protein degradation (Eytan et al., 1989; Driscoll & Goldberg, 1990; Rivett, 1990; Heinemeyer et al., 1991).

The proteinase has a hollow cylindrical structure, composed of at least 24 subunits. The number of different types of polypeptide, each with a molecular mass in the range 22–34 kDa, depends on the species. The simplest proteinase, composed of only two different types of subunits, has been isolated

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[1] Abbreviations: tBoc, *tert*-butyloxycarbonyl; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LLE1, high-affinity site assayed at 0.1 mM LLE-NA; LLE2, lower affinity site(s) assayed at 0.4 mM LLE-NA; LLE-NA, Z-Leu-Leu-Glu-β-naphthylamide; MCP, multicatalytic proteinase; SBzl, thiobenzyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Z, benzyloxycarbonyl.

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from archaebacteria (Dahlmann et al., 1989). MCP purified from higher eukaryotes may contain as many as 25 different polypeptides (Martins de Sa et al., 1986; Rivett & Sweeney, 1991).

The mammalian MCP complex has several distinct types of proteolytic activities (Wilk & Orlowski, 1983). With peptide substrates it can catalyze the cleavage of peptide bonds on the carboxyl side of basic, hydrophobic, or acidic amino acid residues (Wilk & Orlowski, 1980; Rivett, 1985a; McDermott et al., 1991). On the basis of the results of inhibitor studies and mixed-substrate experiments using synthetic peptide substrates (Wilk & Orlowski, 1980; Rivett, 1989b; Orlowski & Michaud, 1989), the three activities are believed to be catalyzed at distinct sites. Multicatalytic proteinase activities catalyzing cleavage of peptide bonds adjacent to basic (usually Arg-X) or hydrophobic (usually Leu-X, Phe-X, or Tyr-X) residues have been described, by analogy, as "trypsin-like" and "chymotrypsin-like" activities, respectively, and it is these two activities of the proteinase which are usually assayed. Although the third distinct MCP activity, that responsible for the cleavage of the synthetic peptide Z-Leu-Leu-Glu-β-naphthylamide and of Glu-X bonds in peptide substrates, was identified in early studies of the complex (Wilk & Orlowski, 1983), rather few subsequent studies of the proteinase have included a substrate for it, and until recently, it has not been well characterized. This activity has been referred to as the "peptidylglutamyl–peptide bond hydrolyzing activity", but as demonstrated here, Asp-X bonds are also cleaved by MCP.

This report describes important and previously unrecognized properties of the MCP complex. Since the first account of the sigmoidal dependence of velocity on substrate concentration, suggesting cooperative interactions within the MCP complex (Rivett et al., 1990), there have been other reports of similar findings (Arribas & Castano, 1990; Orlowski et al., 1991). However, a more detailed analysis of these effects shows major differences from the results of the other studies and demonstrates novel and important aspects of multicatalytic proteinase kinetics. First, the results presented here demonstrate the presence of a high-affinity peptidylglutamyl–peptide hydrolase activity which obeys Michaelis–Menten kinetics. In contrast to the inhibition of activity measured at high substrate concentrations, activity at the high-affinity site is stimulated by the serine protease inhibitor 3,4-dichloroisocoumarin, and there are also differences in the effects of divalent metal ions and of KCl. Second, our results at high substrate (Z-Leu-Leu-Glu-β-naphthylamide) concentrations demonstrate either an allosteric activation of the Glu-X hydrolase activity or positive cooperativity between multiple sites. Third, we observe that Mn^{2+} ions promote the formation of an activated form of the complex. A preliminary account of this work has been communicated previously (Djaballah & Rivett, 1991).

Experimental Procedures

Materials. Rats (Wistar) were obtained from the University of Leicester Biomedical Services Unit. Z-Leu-Leu-Glu-β-naphthylamide, bovine serum albumin, antipain, leupeptin, 3,4-dichloroisocoumarin, Chelex-100, *N*-ethylmaleimide, and methylmethanethiosulfonate were purchased from Sigma Chemical Co. (U.K.). *N*-tBoc-Ala-Ala-Asp-SBzl and Glu-β-naphthylamide were a kind gift from Dr. Martin Poe (Merck, Sharp & Dohme Research Laboratories, Rahway, NJ). All reagents were of analytical grade.

Purification of the Multicatalytic Proteinase. The purification of MCP was carried out from fresh rat livers as described previously (Rivett & Sweeney, 1991). The purified enzyme was stored, at −20 °C, in 50 mM potassium phosphate buffer, pH 7.0, containing 10% glycerol (v/v), 1 mM 2-mercaptoethanol, and 0.1 mM EDTA at a concentration of 1 mg/mL. Protein concentrations were determined by the method of Bradford (1976) using the reagent from Bio-Rad with bovine serum albumin as the standard. SDS–PAGE was performed with 15% polyacrylamide gel (Laemmli, 1970). Gels were stained for protein with Coomassie brilliant blue R. Nondenaturing PAGE gels were prepared as described (Rivett, 1985a), and proteinase activity was detected under a UV light following incubation of gels at 37 °C with 0.1 or 0.4 mM LLE-NA under the conditions described below.

Determination of Proteinase Activity. The Glu-X hydrolase activity was measured with Z-Leu-Leu-Glu-β-naphthylamide (LLE-NA) or Glu-β-naphthylamide. The reaction mixture (200 μL) contained substrate (concentrations indicated in figure and table legends) and 2 μg of MCP in 0.05 M Hepes/KOH buffer, pH 7.5. Reaction mixtures were incubated for 20 or 30 min at 37 °C, stopped by the addition of 0.3 mL of ethanol, and diluted to a volume of 2.5 mL with H_2O. The released β-naphthylamine was measured by a fluorometric procedure (Dahlmann et al., 1985) rather than by the indirect diazotization procedure (Wilk & Orlowski, 1980). A Perkin-Elmer LS-3B fluorescence spectrometer was used with an excitation wavelength of 333 nm and an emission wavelength of 450 nm. A calibration curve for free β-naphthylamine estimation was prepared by digesting Z-Leu-Leu-Glu-β-naphthylamide with the *Staphylococcus aureus* strain V8 proteinase, in 50 mM ammonium bicarbonate buffer, pH 7.8, under which conditions it is specific for glutamyl bonds (Drapeau, 1977). For the substrate variation experiments, the concentration of dimethyl sulfoxide was kept constant at 6%. No difference was observed when activity in 6% dimethyl sulfoxide was compared with activity in 1% (0.1 mM LLE-NA) or 4% (0.4 mM LLE-NA) dimethyl sulfoxide.

MCP activity was also measured with a peptide thioester substrate, Boc-Ala-Ala-Asp-SBzl, as described by Poe et al. (1988). The reaction mixture (200 μL) contained 0.4 mM substrate and 2 μg of the purified enzyme in 0.05 M Hepes/KOH buffer, pH 7.5. Incubations were for 30 min at 37 °C. The released 4-nitrobenzenethiol was quenched by the addition of 0.5 mM DTNB, and the increase in absorbance was measured at 412 nm using a Perkin-Elmer Lambda 5 spectrophotometer. Absorbance increases were converted to enzymatic rates using an extinction coefficient of 13 600 cm^{-1} M^{-1}. The same volume of DTNB was added to the reference cell in order to correct for the background reaction of DTNB with the sulfhydryl groups on the proteinase and the nonenzymatic hydrolysis of the thiobenzyl ester. Substrate stock solutions were made up in dimethyl sulfoxide and stored at −20 °C.

Inhibition Studies. MCP was preincubated for 30 min at 25 °C in 50 mM Hepes/KOH, pH 7.5, with protease inhibitors prior to 20-fold dilution and assay for 20 min at 37 °C. For studies with 3,4-dichloroisocoumarin, MCP was dialyzed before use to remove 2-mercaptoethanol which inactivates the inhibitor. Stock solutions of 3,4-dichloroisocoumarin (10 mM) were made up in dimethyl sulfoxide. For studies with divalent metal ions, 50 mM Hepes/KOH buffer, pH 7.5, was pretreated with Chelex-100.

Results

Proteinase Purification and Assays. The purification protocol described in Experimental Procedures yielded an apparently homogeneous preparation of the rat liver proteinase. Results of a typical purification are shown in Table I. Pep-

Table I: Purification of the Multicatalytic Proteinase from Rat Liver[a]

step	volume (mL)	protein (mg/mL)	total protein (mg)	LLE1 total act. (nmol/min)	LLE1 sp act. (nmol min^{-1} mg^{-1})	LLE2 total act. (nmol/min)	LLE2 sp act. (nmol min^{-1} mg^{-1})
(1) supernatant	870	19	16530	8265	0.5 (1)	10550	0.6 (1)
(2) ammonium sulfate fractionation	270	30	8100	2430	0.3 (1)	4860	0.6 (1)
(3) DEAE-cellulose chromatography	110	4.4	484	1160	2.4 (5)	968	2.0 (4)
(4) anion-exchange FPLC	88	0.4	38	152	4.0 (8)	418	11.0 (18)
(5) Superose 6 gel filtration	8	1.6	13	196	15.0 (30)	884	68.0 (114)
(6) anion-exchange FPLC (Mono Q)	3	3.7	11	187	17.0 (34)	1540	140.0 (234)

[a] Purification of the proteinase was from 115 g of rat liver. Activities were determined with either 0.1 mM LLE-NA (LLE1) or 0.4 mM LLE-NA (LLE2) in assays as described under Experimental Procedures. Samples from steps 2–4 and 6 were dialyzed against low salt containing buffer prior to the determination of specific activities. Values in parentheses indicate the purification achieved.

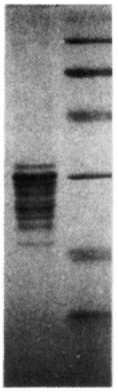

FIGURE 1: SDS–PAGE gel of the purified rat liver multicatalytic proteinase. Lane 1, purified MCP; lane 2, molecular mass markers (phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

tidylglutamyl–peptide hydrolase activity was assayed at both 0.1 mM (LLE1) and 0.4 mM (LLE2) LLE-NA (see below). The purification of LLE1 and LLE2 activities was comparable in steps 1–3, but not from step 4 onward. The overall purification of LLE1 was only around 34-fold as compared to 234-fold for LLE2. Also, an increase in total activity of LLE2 was observed after step 4, suggesting activation of the enzyme, possibly as a result of the removal of inhibitory or competing proteins. Figure 1 shows the characteristic pattern of the low molecular mass polypeptides (20–35 kDa) seen on SDS–PAGE gels of purified MCP. On nondenaturing gels, activities at 0.1 and 0.4 mM LLE-NA were both found to be associated with the single protein band.

The purified proteinase gave specific activities of 17 and 140 nmol min^{-1} mg^{-1} for LLE1 and LLE2, respectively. Activity was linear with protein concentration for LLE1, but for LLE2 it was linear only up to 4 μg/assay. The specific activities using different synthetic peptide substrates with either Glu or Asp in the P1 position are summarized in Table II. Activities of the purified proteinase were not stimulated by 1 mM MgATP but could be activated by low concentrations (e.g., 0.01%) of SDS.

Activity with Varying Substrate Concentrations. The range of substrate concentrations used for assays was limited by the solubility of the peptide substrate. The solubility limit of LLE-NA was investigated by measuring turbidity due to insoluble peptide at 340 nm. Solubility was both pH- and temperature-dependent (results not shown), and the limit of interest was 0.6 mM LLE-NA at 37 °C in 0.05 M Hepes/KOH buffer, pH 7.5.

Table II: MCP Substrates for Cleavage on the Carboxyl Side of Acidic Residues

substrate	concn (mM)	sp act.[a] (nmol min^{-1} mg^{-1})
Z-Leu-Leu-Glu-β-NA	0.4	165 ± 73
Glu-β-NA	0.4	13 ± 3
Boc-Ala-Ala-Asp-SBzl	0.4	41 ± 3

[a] Hydrolysis rates were measured in 50 mM Hepes/KOH buffer, pH 7.5, containing 6% dimethyl sulfoxide at 37 °C with 2 μg of purified MCP as described under Experimental Procedures. The mean ± SD was calculated from experiments with at least two different MCP preparations.

The kinetics of the Glu-X hydrolase activity with Z-Leu-Leu-Glu-β-naphthylamide as the substrate are very complex as illustrated by the plot of activity as a function of substrate concentration (Figure 2). The first part of the curve (substrate range 0–100 μM) was less variable (standard deviation less than 5%) and followed normal Michaelis–Menten kinetics, with an apparent K_m value of 114 μM and an estimated V_{max} of 33 nmol min^{-1} mg^{-1}. However, the second part of the curve (substrate range 0.2–0.6 mM) yielded a sigmoidal curve (Figure 2). Because saturation was not reached at substrate concentrations below the solubility limit of the substrate, it was not possible to estimate $K_{0.5}$ from the inflection point of the curve. Double-reciprocal plots (not shown) were biphasic with an upward concavity, indicating the presence of positive cooperative effects. The above observations suggest that the peptidylglutamyl–peptide bond hydrolase activity is composed of two components. Therefore, further assays were carried out at both 0.1 mM (LLE1) and 0.4 mM (LLE2) LLE-NA.

The Effect of pH and KCl and the Thermal Stability of LLE1 and LLE2. The effect of pH on peptidylglutamyl–peptide hydrolase activity was measured in the pH range 6.5–9.0 in three different buffer systems with 0.1 mM (LLE1) and 0.4 mM (LLE2) LLE-NA as illustrated in Figure 3. The pH optimum for the LLE2 activity was broad in the range 7–7.5. That for the LLE1 activity was pH 6.5 in the Tris-HCl buffer system but pH 7.0 in Hepes/KOH or Bis-Tris propane buffers. The sharp decline in LLE2 activity above pH 8.0 is unlikely to be due to instability of the proteinase (Rivett, 1985a) but may reflect the loss of allosteric effects at higher pH values (Kurganov, 1982).

Examination of the LLE1 and the LLE2 activities as a function of KCl concentration also yielded a clear distinction between the two activities (Figure 4). The LLE 1 activity is slightly inhibited by an increase in KCl concentration, whereas the LLE2 activity was found to be sensitive to KCl. Approximately 70% activity was lost at a KCl concentration of 25 mM, and no further inhibition was observed up to 50 mM KCl. The two activities also respond differently to heat

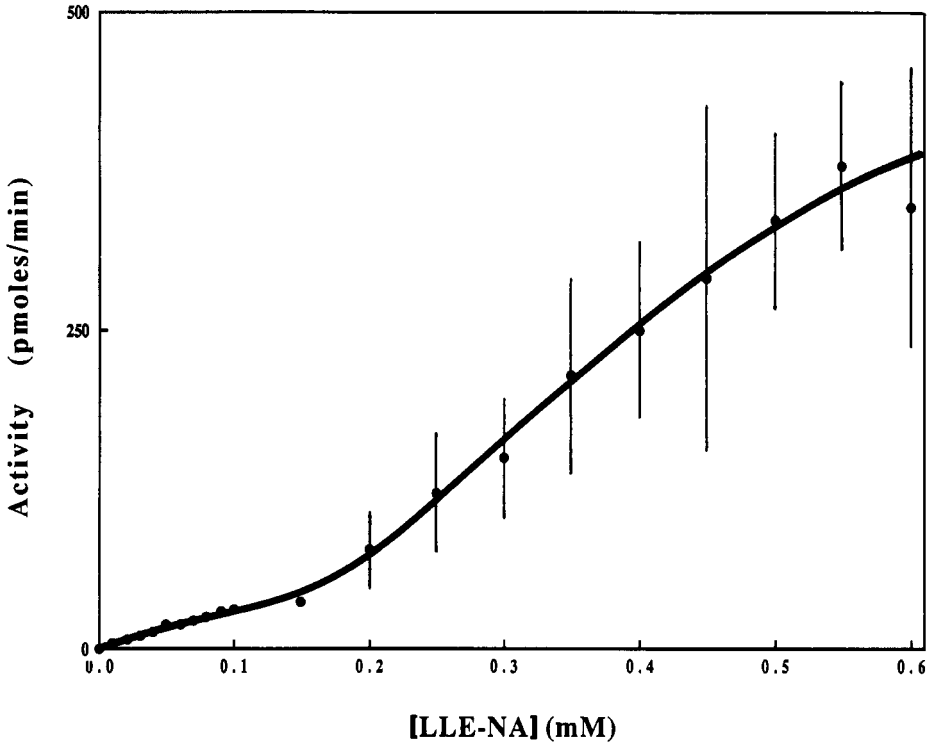

FIGURE 2: MCP activity with varying Z-Leu-Leu-Glu-β-naphthylamide concentrations. Hydrolysis rates were measured in 50 mM Hepes/KOH buffer, pH 7.5, containing 6% dimethyl sulfoxide as described under Experimental Procedures. The error bars represent standard deviations from at least seven separate experiments. Standard deviations less than 5% are not shown.

Table III: Effects of Divalent Metal Ions and EDTA on the Peptidylglutamyl-Peptide Hydrolase Activity

compd	concn (mM)	LLE1 act.[a] (% of control)	LLE2 act.[a] (% of control)
$MgCl_2$	1.0	229 ± 55	79 ± 39
$CaCl_2$	1.0	188 ± 50	111 ± 65
$MnCl_2$	1.0	306 ± 44	192 ± 100
$CdCl_2$	1.0	34 ± 9	10 ± 5
$ZnCl_2$	1.0	28 ± 11	8 ± 6
$CoCl_2$	1.0	88 ± 16	93 ± 55
EDTA	1.0	64 ± 10	106 ± 16
EDTA	10.0	44 ± 4	96 ± 3

[a] Activities were determined as described under Experimental Procedures with 0.1 mM (LLE1) and 0.4 mM (LLE2) LLE-NA. Values are given as the mean ± SD for six separate experiments.

Table IV: Effect of Inhibitors on the Peptidylglutamyl-Peptide Bond Hydrolase Activity

compd	concn	activity[b] (% of control) LLE1	LLE2
3,4-DCI[a]	20 μM	173 ± 61	34 ± 2
NEM[a]	0.1 mM	98 ± 2	108 ± 4
	1.0 mM	84 ± 8	97 ● 3
MMTS[a]	0.1 mM	95 ± 3	100 ± 3
	1.0 mM	92 ± 4	88 ± 6
antipain	0.01 mg/mL	71 ± 10	102 ± 2
leupeptin	0.01 mg/mL	66 ± 4	101 ● 3

[a] Abbreviations: 3,4-DCI, 3,4-dichloroisocoumarin; NEM, *N*-ethylmaleimide; MMTS, methylmethanethiosulfonate. [b] Preincubations were at 25 °C and contained 0.1 mg/mL proteinase. Aliquots were transferred to incubation mixtures at 37 °C for measurement of remaining activity as described under Experimental Procedures. Values are given as the mean ± SD of at least three separate experiments.

treatment, with LLE2 being more stable than LLE1 (Figure 5).

Effect of Divalent Metal Ions. Table III summarizes the effect of selected divalent metal ions and EDTA on the LLE1 and the LLE2 activities. There is a clear difference in their response to metal ions and to EDTA. The LLE1 activity was activated by 1 mM $MnCl_2$, $MgCl_2$, and $CaCl_2$ and was inhibited by $LiCl_2$, $CdCl_2$, $CoCl_2$, and EDTA. The LLE2 activity, on the other hand, was stimulated up to 2-fold by $MnCl_2$, $CoCl_2$, and $CaCl_2$, but EDTA had little effect. Higher concentrations (≥5 mM) of $MnCl_2$ were found to inhibit both LLE1 and LLE2 activities (data not shown).

Examination of Glu-X hydrolase activity in the presence of 1 mM $MnCl_2$ yielded a curve similar to that obtained in the absence of the metal ion except that saturation was achieved at the higher end of substrate range (see Figures 2 and 6). The nonsigmoidal part of the curve obeyed Michaelis–Menten kinetics with a more effective binding of the substrate, giving a K_m value of 67 μM and a V_{max} of 28 nmol min^{-1} mg^{-1} (Figure 6). Analysis of the sigmoidal part of the curve (averaged from six experiments carried out in the presence of manganese) gave a $K_{0.5}$ of 0.28 mM, a V_{max} of 302 nmol min^{-1} mg^{-1}, and a Hill coefficient, n_H, of 5.1 (Figure 7).

Activity with 3,4-Dichloroisocoumarin-Treated Enzyme. 3,4-Dichloroisocoumarin, a mechanism-based inhibitor for serine-type proteinases (Harper et al., 1985), inactivated the LLE2 activity. A total of 70% of the activity was lost after 30 min of preincubation with 20 μM 3,4-dichloroisocoumarin. However, LLE1 activity was stimulated up to 2-fold by the same treatment (Table IV), confirming that the LLE1 and the LLE2 could be two separate components of the peptidylglutamyl-peptide hydrolase activity. Thiol-reactive reagents such as *N*-ethylmaleimide and methylmethanethiosulfonate had little effect on either the LLE1 or the LLE2 activities (Table IV). The peptide aldehydes antipain and leupeptin slightly inhibited the LLE1 activity but had no inhibitory effects on the LLE2 activity.

When the proteinase was pretreated with 3,4-dichloroisocoumarin to inhibit LLE2, the substrate-dependence profile did not display the apparent cooperative effect but showed only the rectangular hyperbola for LLE1 (Figure 8). Analysis of

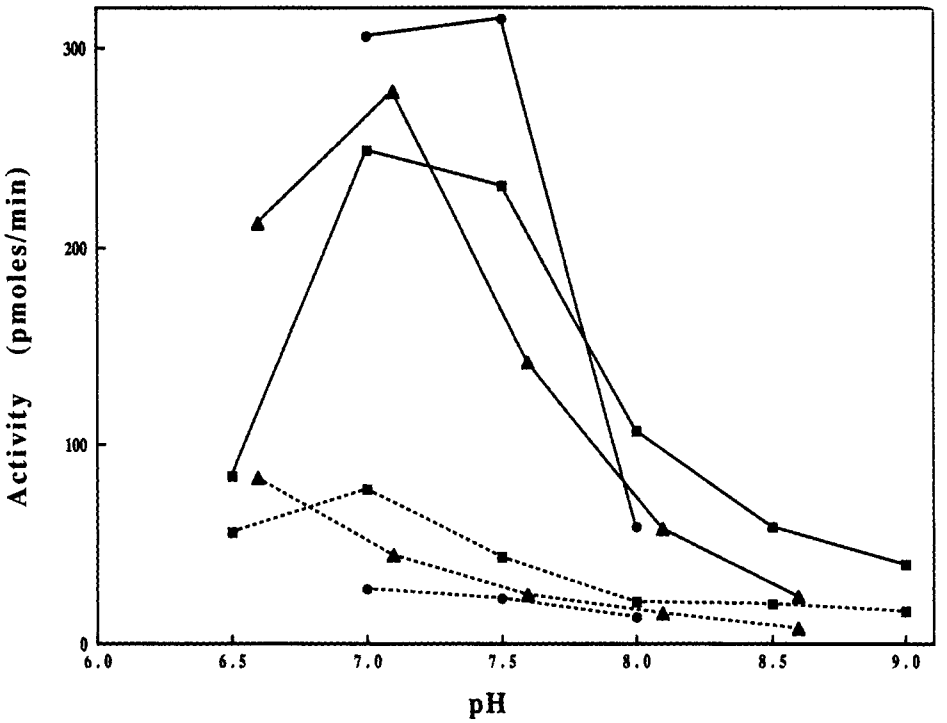

FIGURE 3: pH profile of the peptidylglutamyl-peptide activity. Hydrolysis rates of 0.1 mM (---) and 0.4 mM (—) LLE-NA were measured in 50 mM Hepes/KOH (●), 50 mM Tris-HCl (■), or Bis-Tris propane/HCl (▲) as described under Experimental Procedures.

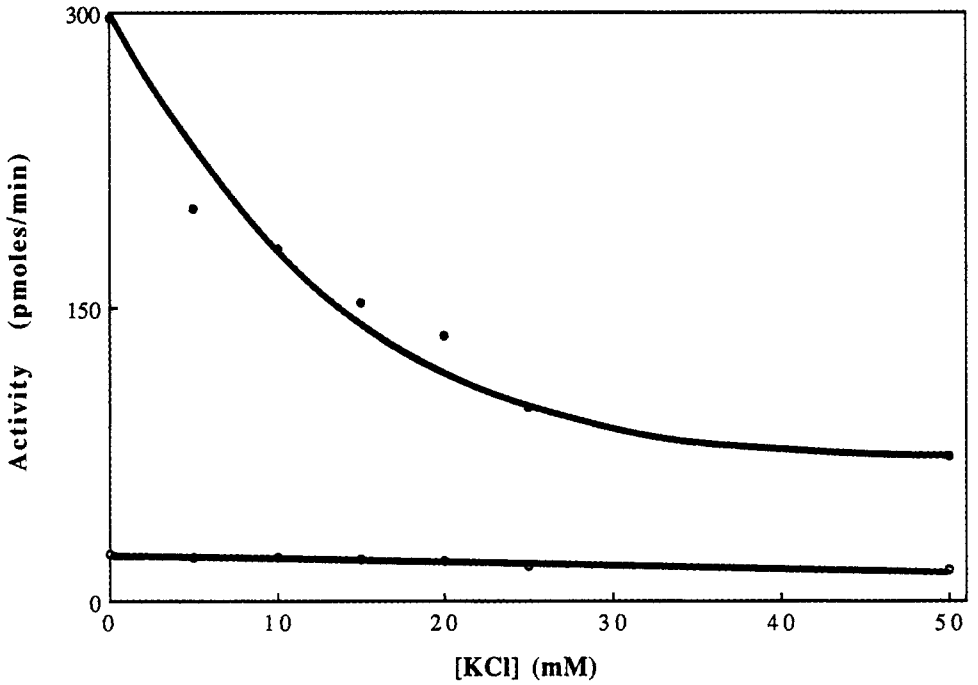

FIGURE 4: Effect of potassium chloride on the peptidylglutamyl-peptide hydrolase activity. Hydrolysis rates were measured in 50 mM Hepes/KOH Chelex-treated buffer, pH 7.5, including appropriate KCl concentrations with either 0.1 mM (○) or 0.4 mM (●) LLE-NA as substrate.

the hyperbola gave a K_m of 70 μM and a V_{max} of 79 nmol min^{-1} mg^{-1}. The kinetic parameters are summarized in Table V.

Discussion

Purification of the multicatalytic proteinase by the procedure described yields apparently pure preparations which show the characteristic set of proteins on SDS-PAGE gels. The MCP complex can account for up to 0.6% of the soluble protein in human cells (Hendil, 1988), and in the rat, the highest levels are found in liver (Rivett & Sweeney, 1991). The difference in the relative activity measured at 0.1 and 0.4 mM LLE-NA at various stages of the purification probably reflects the problems of accurately assaying MCP activity in crude preparations which occur as a result of interference by other proteases as well as by other proteins. The peptidase assay is reliable for the purified proteinase although LLE-NA hydrolysis is only linear with protein concentration over a limited range. Activity is inhibited by low concentrations of casein (unpublished results), in agreement with published results with MCP isolated from bovine pituitary and human brain (Wilk & Orlowski, 1983; Orlowski et al., 1991; Mc Dermott et al., 1991). The specific activity of the purified rat liver MCP with LLE-NA as a substrate is similar to values given for the complex from bovine pituitary (Orlowski & Michaud, 1989),

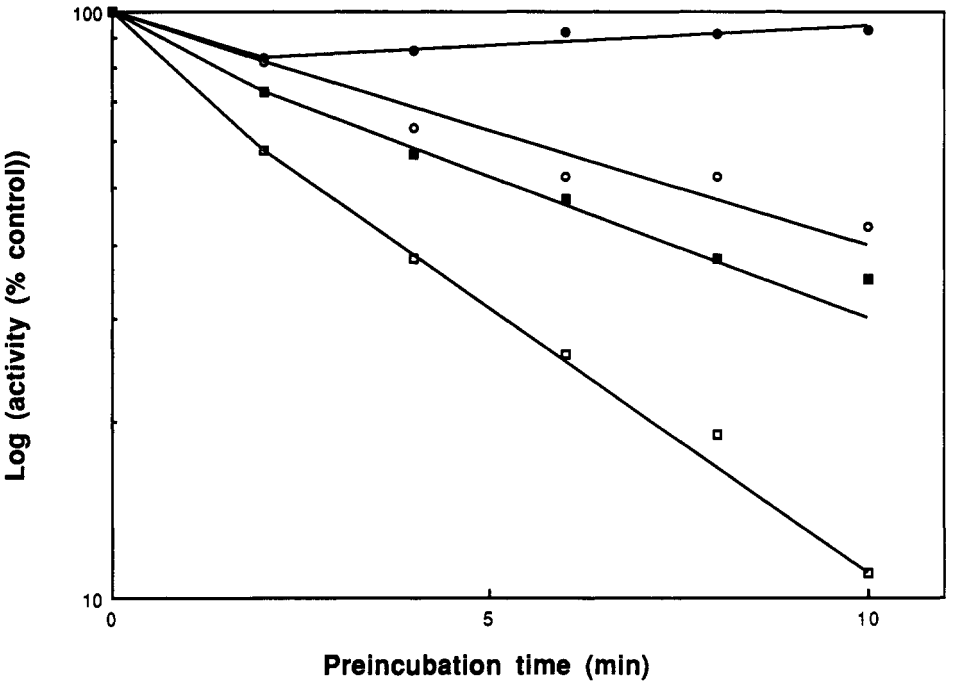

FIGURE 5: Thermal stability of peptidylglutamyl–peptide hydrolase activities. Hydrolysis of 0.1 mM (closed symbols) and 0.4 mM (open symbols) LLE-NA was assayed following preincubation of MCP (0.2 mg/mL) at 55 °C (●, ○) or 60 °C (■, □).

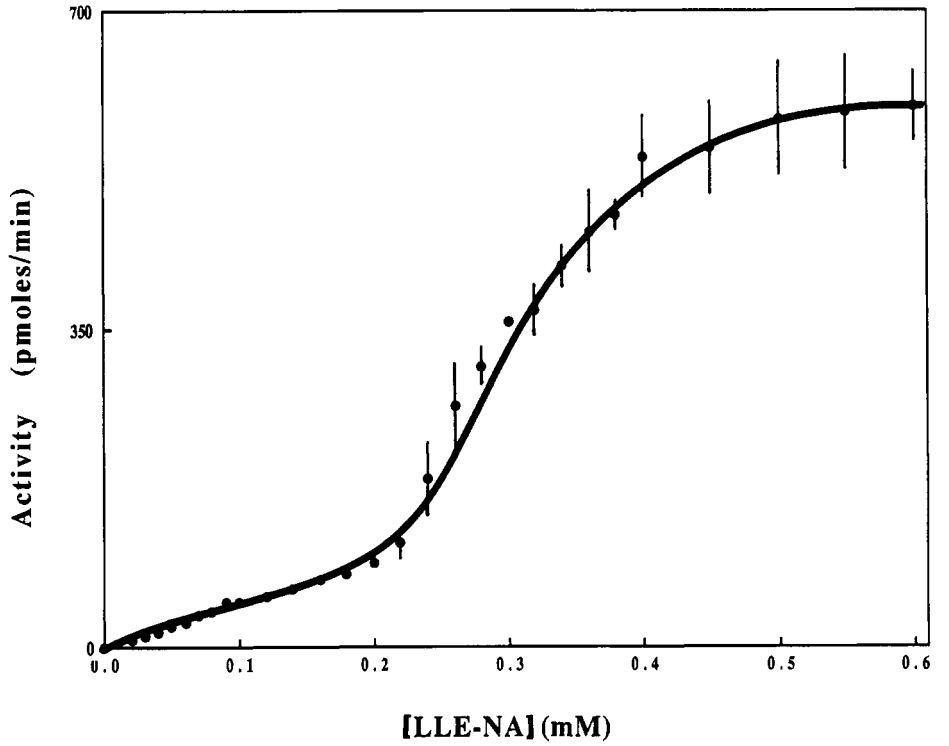

FIGURE 6: Hydrolysis of Z-Leu-Leu-Glu-β-naphthylamide by MCP in the presence of $MnCl_2$ as a function of substrate concentration. Hydrolysis rates were measured in 50 mM Hepes/KOH Chelex-treated buffer, pH 7.5, containing 1 mM $MnCl_2$ and 6% dimethyl sulfoxide, as described under Experimental Procedures. The error bars represent standard deviations from six separate experiments. Standard deviations less than 5% are not shown.

bovine lens (Wagner et al., 1985), and human brain (McDermott et al., 1991) in assays under comparable conditions. Apparent discrepancies in the literature values can largely be explained by the differences in substrate (LLE-NA) concentration and buffer composition.

The peptidylglutamyl–peptide hydrolase activity of rat liver MCP, unlike the chymotrypsin-like and trypsin-like activities (Rivett, 1989b), can be assayed with a single amino acid derivative such as Glu-β-naphthylamide. Z-Ala-Ala-Asp-SBzl, an example of a new class of protease substrates (Odake et al., 1991), was also found to be a substrate for MCP, clearly demonstrating for the first time that the enzyme can catalyze cleavage on the carboxyl side of Asp residues.

At concentrations of LLE-NA below 0.1 mM, the enzyme complex obeyed Michaelis–Menten kinetics, suggesting the presence of one type of relatively high-affinity site (LLE1) with a K_m value of around 100 μM. In contrast to our results, Arribas and Castano (1990) suggested the presence of a cooperative component with a similar K_m value. The LLE1 type of catalytic site has probably gone unnoticed in other studies

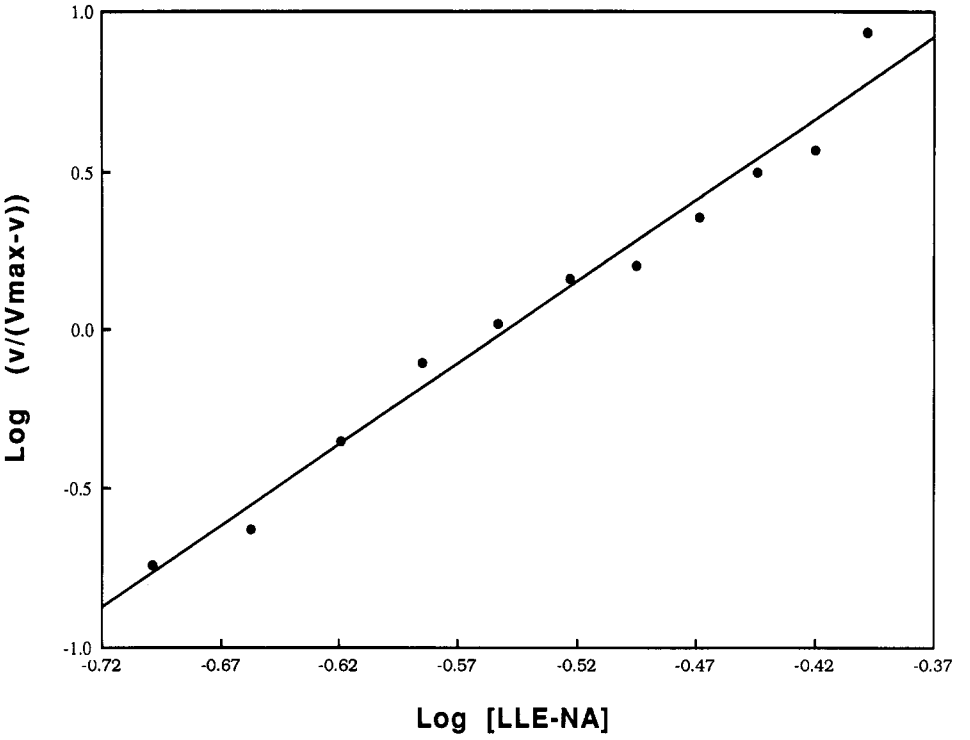

FIGURE 7: Hill plot of the data from Figure 6. V_{max} was estimated from Figure 6 and log $(v/(V_{max} - v))$ values calculated for each of the data points within the appropriate substrate concentration range. The Hill coefficient calculated from the slope is 5.1.

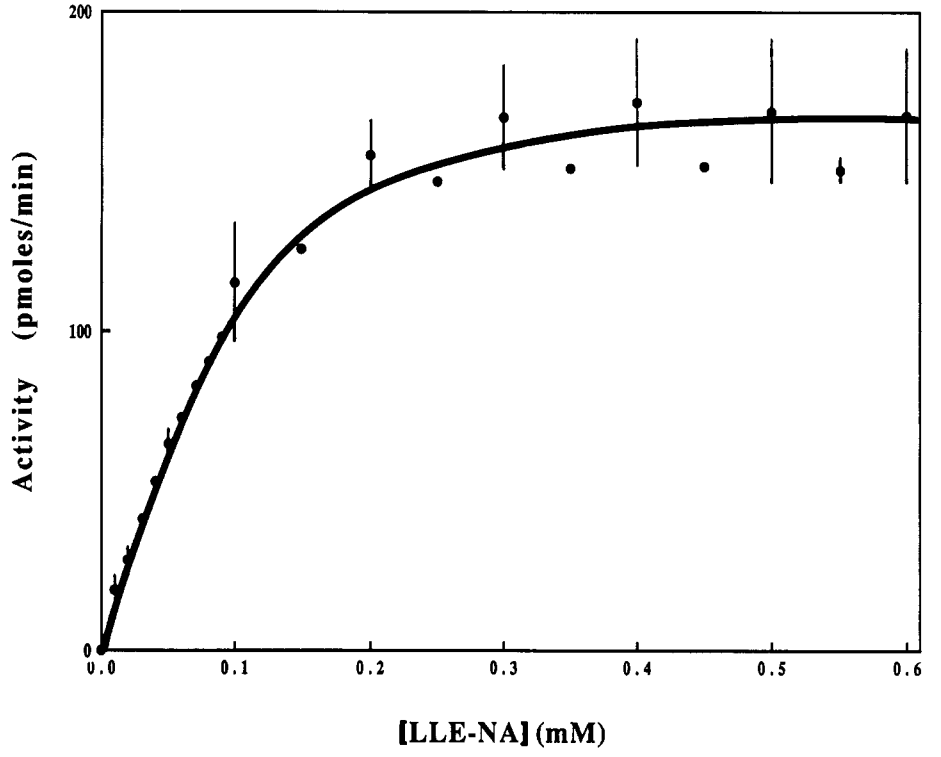

FIGURE 8: Effect of 3,4-dichloroisocoumarin on the rate of hydrolysis of Z-Leu-Leu-Glu-β-naphthylamide as a function of substrate concentration. The inactivation reaction was initiated by addition of 20 μM inhibitor to the dialyzed enzyme solution, and the residual activity measured as described under Experimental Procedures. The final concentration of dimethyl sulfoxide was 6%. The error bars represent standard deviations of at least three separate experiments. Standard deviations less than 5% are not shown.

because of the high LLE-NA concentrations (up to 0.8 mM) used for assays (Orlowski & Michaud, 1989; Orlowski et al., 1991). At high LLE-NA concentrations, unlike the case for degradation of other types of peptide substrate (Rivett, 1989b; McGuire & DeMartino, 1986), MCP does not obey Michaelis–Menten kinetics but gives rise to sigmoidal velocity versus substrate concentration curves.

The activity with high (e.g., 0.4 mM) concentrations of LLE-NA was somewhat variable in different experiments, but this variation could be reduced considerably by the addition of $MnCl_2$, which was found not only to stimulate peptidyl-glutamyl–peptide hydrolase activity but also to reduce the concentration at which saturation with substrate was observed. This latter observation allowed calculation of a Hill coefficient

Table V: Kinetic Parameters of the Noncooperative Component of the Peptidylglutamyl-Peptide Bond Hydrolase Activity[a]

treatment	K_m[b] (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
normal assay conditions	114	0.357	3130
including 1 mM $MnCl_2$	67	0.296	4420
using 3,4-DCI[c]-treated enzyme	70	0.856	12230

[a] Assays were carried out as described under Experimental Procedures. [b] Kinetic constants were determined using the method of Eisenthal and Cornish-Bowden (1974). [c] Abbreviation: 3,4-DCI, 3,4-dichloroisocoumarin. 3,4-DCI-treated enzyme was assayed in the absence of $MnCl_2$.

of 5.1, which can be explained either by the presence of at least five relatively low-affinity sites acting in a cooperative manner or by an allosteric effect. In view of the large number of different but related polypeptides associated with the complex (Rivett & Sweeney, 1991), it seems unlikely that many catalytic sites could be equivalent.

The effects of KCl and inhibitors provide further evidence for the presence of two distinct types of catalytic component for Glu-X hydrolase activity. The activity with 0.4 mM LLE-NA was found to be inhibited by low concentrations of KCl (e.g., 10 mM), whereas at 0.1 mM LLE-NA there is relatively little effect even at 50 mM KCl. Also, the serine protease inhibitor, 3,4-dichloroisocoumarin (Harper et al., 1985), inhibits degradation of LLE-NA at high substrate concentrations (LLE2) (Orlowski & Michaud, 1989) but stimulates the activity at low concentrations ($\leq$0.1 mM, LLE1). The latter observation allows activity at the high-affinity site to be studied in the absence of LLE2 activity. In our experiments, it was not possible to reach V_{max} for the high-affinity site without inhibiting the LLE2 component, suggesting some negative interaction between LLE1 and LLE2 components.

The latter observations provide the best evidence that the two activities are catalyzed within the same particles. Both LLE1 and LLE2 are associated with a single peak at the final step of the purification and with the single protein band on nondenaturing gels. Electron microscopy of negatively stained preparations also suggests a homogeneous population. However, none of these observations can completely rule out the possibility that the proteinase preparation contains a mixture of similar but nonidentical particles.

The above conclusions concerning the cooperative effect differ from those of two recently published studies, both of which addressed the effects of SDS on the catalytic properties of the complex. Arribas and Castano (1990) suggested the presence of two components but in contrast to the results presented here suggested cooperativity of the higher affinity component, while Orlowski et al. (1991) have determined a Hill coefficient of 2.2–2.4. There may be differences in the kinetic properties of MCP isolated from rat liver and bovine pituitary. Another possible explanation of the discrepancies is the dependence of the Hill plot on the accurate determination of V_{max}. The improvement in the data obtained by addition of manganese ions, as well as a large number of data points averaged from six separate experiments, allowed us to calculate the Hill coefficient of 5.1.

Stimulation of peptide hydrolase activity by calcium and cobalt ions is not unusual for proteases. Degradation of α_2-crystallin by lens neutral endopeptidase (MCP) has been reported to be stimulated by calcium ions, but this effect apparently decreases with purification of the complex (Ray & Harris, 1986). Of the divalent metals ions tested in this study, manganese was the most effective stimulator of Glu-X hydrolase activity. Since the requirement for metal ion is not absolute, it seems likely that manganese ions play some structural role.

MCP is now believed to play a major role in nonlysosomal pathways of intracellular protein breakdown. There are several kinetic advantages of the location of several distinct proteolytic centers within a single proteinase molecule. These include the cleavage of a variety of different peptide bonds and possible substrate channeling between sites (Dick et al., 1991) as well as allosteric activation or positive cooperativity. Such properties, which should be advantageous for the rapid and complete degradation of intracellular proteins, are further evidenced by the lack of intermediates detected in the degradation of protein substrates by MCP (Rivett, 1985b). It is not clear to what class of proteinase the new LLE1 site belongs. It is not inhibited by 3,4-dichloroisocoumarin, it is unaffected by several thiol-reactive reagents, and the metal ion requirement is not absolute like that of metalloproteinases. Complete classification of this proteolytic site awaits further structural analysis.

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Use of Azidobestatin as a Photoaffinity Label To Identify the Active Site Peptide of Leucine Aminopeptidase†

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ABSTRACT: Aminopeptidases catalyze the hydrolysis of amino acid residues from the amino terminus of peptide substrates. They are found in most cells and tissues, and their activity has been implicated in myriad fundamental biochemical and physiological processes. Nevertheless, little is known about the structure of the aminopeptidase active sites. Beef lens leucine aminopeptidase (blLAP) can be considered prototypical of many enzymes in this family of peptidases. Bestatin, [(2*S*,3*R*)-(3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine] is a nonhydrolyzable substrate analogue of a peptide, PheLeu, which is rapidly cleaved by blLAP. Bestatin incorporates elements of the putative tetrahedral intermediate, and this results in a $>10^5$-fold enhancement of binding relative to analogous peptides. Bestatin is the most tightly bound inhibitor of many aminopeptidases. Bestatin was successively converted to nitrobestatin, *p*-aminobestatin, [^{3}H]-*p*-aminobestatin, and finally [^{3}H]-*p*-azidobestatin (pAB). Like bestatin, pAB is a slow binding inhibitor of LAP (K_i*, the dissociation constant for the final complex, = $\sim 4 \times 10^{-9}$; K_i, the dissociation constant for the initial collision complex, = $\sim 10^{-8}$). The $t_{1/2}$ for binding of 2×10^{-8} M and 8×10^{-8} M bestatin are ~60 min and ~38 min, respectively. pAB, nitrobestatin, bestatin, and physiological peptides appear to bind in the same site, the first three with similar avidity. In the dark, pAB and bestatin protect low concentrations of the enzyme against inactivation upon extensive dialysis. The $t_{1/2}$ for photoactivation of pAB is approximately 3 s. Irradiation of blLAP for such short periods of time resulted in insignificant change in activity. blLAP which was placed in 254-nm light in the presence of pAB was inactivated significantly. Treatment of photolabeled blLAP with trypsin produces only two peptides. Autoradiography and scintillation counting indicate that the active site is in the peptide which includes residues 138–487. Treatment of the same blLAP with hydroxylamine produces two different peptides, with the active site in the peptide 323–487. This indicates that the active site is in the carboxyl-terminal one-third of the protomer. It is likely that this photoaffinity label will be useful in identifying active sites in other aminopeptidases as well.

Aminopeptidases catalyze the hydrolysis of amino-terminal amino acid residues from peptide substrates. These enzymes are widely distributed throughout the plant and animal kingdoms, and most have broad specificity. Aminopeptidase catalysis occurs on cell surfaces, in cytoplasm, and within various cellular compartments, and several forms of these enzymes have been found in many tissues or cells (Ahmad & Ward, 1990; Chang & Smith, 1989; Ledeme et al., 1983; McDonald & Barrett, 1986; Oettgen & Taylor, 1985; Stirling et al., 1989; Taylor et al., 1982a, 1983, 1984a,b; Watt & Yip, 1989, and references cited within). Despite their ubiquitous distribution and myriad physiological roles (see Discussion), structural information regarding these enzymes is scanty. There has been no indication of the sites involved in substrate or inhibitor binding by covalent attachment of an active site label.

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