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Characterization of the active site of human multicatalytic proteinase

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The activity of multicatalytic proteinase against synthetic substrates and the kinetics of its inhibition by a range of class-specific inhibitors have been investigated. The enzyme was found to have a broader pH activity profile than previously noted, being active against succinyl-Ala-Ala-Phe-7-amino-4-methylcoumarin optimally at pH 4.5 and against benzyloxycarbonyl-Gly-Gly-Arg-7-amino-4-methylcoumarin optimally at pH 10.5. Neither activity was inhibited by the class-specific inhibitors 1,10-phenanthroline, EDTA, pepstatin, di-isopropyl fluorophosphate, peptidyl chloromethanes, peptidyl diazomethanes or L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64), indicating that the enzyme is not a typical metallo-, aspartic, serine or cysteine proteinase. Inhibition by $HgCl_2$, iodoacetamide and *N*-ethylmaleimide suggests that free thiols are necessary for the enzyme to maintain activity, but that these thiols are not particularly reactive as is the case for cysteine proteinases of the papain superfamily. The peptidyl aldehydes chymostatin and leupeptin were found to be reversible inhibitors of multicatalytic proteinase. Chymostatin inhibited activity against succinyl-Ala-Ala-Phe-7-amino-4-methylcoumarin at pH 4.5 (K_i 160 ± 22 μM) whereas leupeptin (200 μM) was not inhibitory. Inhibition of activity against benzyloxycarbonyl-Gly-Gly-Arg-7-amino-4-methylcoumarin by these compounds was more complex, in that they behaved as slow tight-binding inhibitors. k_{on} values were determined to be 12 ± 2 $M^{-1} \cdot s^{-1}$ and 1290 ± 125 $M^{-1} \cdot s^{-1}$ for chymostatin and leupeptin, respectively. The upper limit for K_i values for these two inhibitors was estimated as 5 ± 1.5 μM and 25 ± 5 nM, respectively. The different inhibition characteristics for each substrate were also apparent at an intermediate pH of 8.5, showing that the two activities are distinct. Dichloroisocoumarin, a mechanism-based inhibitor of serine proteinases, did inhibit activity against succinyl-Ala-Ala-Phe-7-amino-4-methylcoumarin with a rate constant of 250 $M^{-1} \cdot s^{-1}$, suggesting that multicatalytic proteinase is an atypical serine proteinase.

INTRODUCTION

Multicatalytic proteinase is a major cytoplasmic enzyme that has been identified in a number of mammalian tissues and cells (Dahlmann *et al.*, 1988). It has a native molecular mass of 650–670 kDa and is composed of a number of subunits of 22–34 kDa (Wilk & Orlowski, 1983). The enzyme has been called 'multicatalytic' in recognition of its ability to degrade synthetic substrates with either glutamic acid, arginine or phenylalanine in P_1 (according to the terminology of Berger & Schecter, 1970). Activity against the latter two types of substrates was inhibited by leupeptin and chymostatin, respectively. The pH optima of these two activities were reported to be 10.0 and 7.5 respectively (Dahlmann *et al.*, 1985), and therefore it was not necessarily true that these activities represented two separate catalytic sites; such different pH optima for basic and neutral substrates has previously been noted for a simple one-chain enzyme with one active site from *Entomeoba histolytica* (Luaces & Barrett, 1988).

There are four recognized classes of proteinases, termed serine, cysteine, metallo- and aspartic (Barrett & McDonald, 1980). These classifications reflect the essential residues for catalysis in the active site of the enzymes. Multicatalytic proteinase has been proposed by different groups to be either a metallo-, serine or cysteine proteinase, based on inhibition characteristics (Zolfaghari *et al.*, 1987; Dahlmann *et al.*, 1985; Tanaka *et al.*, 1986). The most effective inhibitors of this enzyme are mercurial reagents which are specific for free thiols when used at low concentrations (Dahlmann *et al.*, 1985). Di-isopropyl fluorophosphate is an irreversible inhibitor of serine proteinases and has been shown to have variable inhibition effects on multicatalytic proteinase (Tanaka *et al.*, 1986). No studies have yet been published on time-dependent inhibition of multicatalytic proteinase by any irreversible inhibitors.

This report describes the purification and characterization of the enzyme from human liver. The results presented indicate that the enzyme is an atypical serine proteinase with at least two different catalytic sites. An additional pH optimum of 4.5 has also been identified.

MATERIALS AND METHODS

Materials

Peptidyl diazomethane and peptidyl chloromethane inhibitors were gifts from Dr. E. Shaw, Friedrich

Abbreviations used: Z, benzyloxycarbonyl; E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane; -CHN_2, diazomethane; NHMec, 7-amino-4-methylcoumarin; Suc, succinyl.

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Meischer Institut, Basel, Switzerland. Leupeptin, di-isopropyl fluorophosphate, 1,10-phenanthroline, pepstatin, chymostatin, dichloroisocoumarin and E-64 were obtained from Sigma. Suc-Ala-Ala-Phe-NHMec and Z-Gly-Gly-Arg-NHMec were obtained from Cambridge Research Biochemicals.

Purification of enzyme

The purification of human multicatalytic proteinase was based on the method described by Dahlmann *et al.* (1985) for purification of the enzyme from rat muscle. Briefly, human post-mortem liver which had been stored at −20 °C was homogenized in 100 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA and 0.1 % 2-mercaptoethanol. After removal of the insoluble debris by centrifugation, a 20–65 % $(NH_4)_2SO_4$ fractionation was performed. This was then subjected to chromatography on DEAE-Sephacel, Sepharose 6B and f.p.l.c. Mono Q as described previously. Final purification was achieved by applying the sample on to a column of phenyl-Sepharose in 20 mM-Tris/HCl, pH 8.0, containing 500 mM-NaCl. The enzyme was unretarded and trace contaminants retained. Samples from each stage in the purification were subjected to SDS/polyacrylamide-gel electrophoresis as described by Bury (1981).

pH optimum determination

Activity against the substrates Suc-Ala-Ala-Phe-NHMec and Z-Gly-Gly-Arg-NHMec were determined at a range of pH values using buffers of either constant conductivity or constant molarity. Assay mixture contained enzyme (10 μg), substrate (5 μM) and buffer in a total volume of 1 ml. The enzyme was preincubated in assay buffer for 15 min prior to addition of substrate. Incubation was for 30 min at 37 °C and assays were stopped by addition of an equal volume of 1 mM-$HgCl_2$. Fluorescent product released was determined as described previously (Crawford *et al.*, 1988).

Effect of ionic strength and conductivity of buffers

The enzyme was assayed in the presence of a range of buffers at pH 10.0 using Z-Gly-Gly-Arg-NHMec (5 μM) as substrate or in sodium acetate buffers of increasing molarity at pH 4.5 using the substrate Suc-Ala-Ala-Phe-NHMec. The conductivity of the buffers was measured using a Radiometer conductivity meter.

Inhibition studies

Enzyme assays were performed at 37 °C by continuously monitoring release of product. Activity against Suc-Ala-Ala-Phe-NHMec (5 μM) was assayed in 20 mM-sodium acetate buffer, pH 4.5, and activity against Z-Gly-Gly-Arg-NHMec (5 μM) was assayed in 50 mM-diethanolamine/HCl buffer, pH 10.0. When a linear rate of product formation was recorded, inhibitor dissolved in 30 μl of dimethyl sulphoxide was added.

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \qquad (1)$$

For reversible inhibitors (eqn. 1), the reaction was continued until a new linear rate was obtained. K_i was determined by using several concentrations of inhibitor by the method of Henderson (1972).

For slow-binding reversible inhibitors, K_i was determined by preincubating enzyme with a range of concentrations of inhibitor for 16 h before measuring residual activity. k_{on} was determined by measuring release of product continuously with $[I] \gg K_i$. In this case the inhibitors behaved essentially as irreversible inhibitors (Baici, 1988) and inactivation rate constants were determined as described below.

$$E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_{+2}}{\longrightarrow} EI^* \qquad (2)$$

For irreversible inhibitors (eqn. 2), the reaction was continued to monitor decay of activity. The appearance of product progress curves were analysed by the modified method of Tian & Tsou (1982) as described by Crawford *et al.* (1988). When the second-order rate of inactivation determined was dependent upon inhibitor concentration, indicating that the condition $[I] \ll K_i$ was not met, the individual rate constants k_{+2} and K_i were determined by replots of $1/k_{obs.}$ versus [I] as described by Knight (1986).

RESULTS AND DISCUSSION

Purification of enzyme

Multicatalytic proteinase was purified 115-fold with a yield of 47 % (Table 1). Samples from each stage of the purification are shown in Fig. 1. The purified enzyme consisted of a number of subunits of M_r 22000–32000, similar to results seen previously for the enzyme purified from human lung and other species (Zolfaghari *et al.*, 1987; Dahlmann *et al.*, 1985; Tanaka *et al.*, 1986).

Table 1. Purification of human multicatalytic proteinase

Starting material was 100 g of frozen human liver. Activity was measured against Z-Gly-Gly-Arg-NHMec (5 μM) in 50 mM-diethanolamine/HCl buffer, pH 10, at 37 °C. For purification details see the text.

	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (-fold)	Yield (%)
1st supernatant	6120	173.4	0.028	1	100
DEAE-Sephacel	232.2	189.0	0.822	29	111
Sepharose 6B	87.4	156.2	1.79	63	91
Mono Q	35.2	80.2	2.27	80	47
Phenyl-Sepharose	25.2	82.1	3.26	115	47

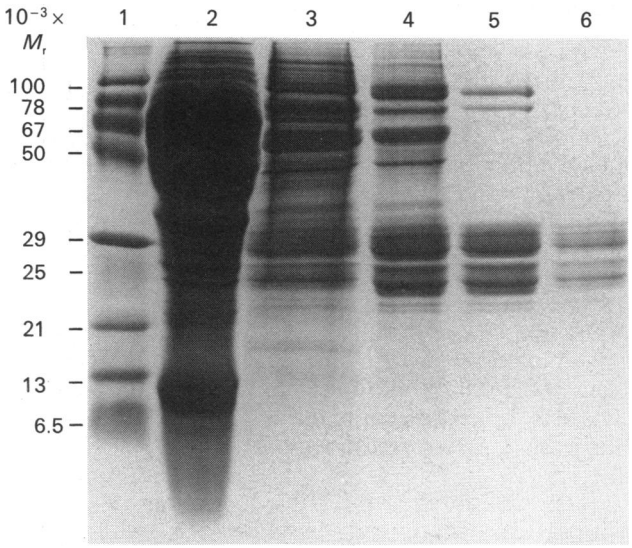

Fig. 1. Purification of multicatalytic proteinase

Samples of protein from each stage of the purification were subjected to SDS/12.5%-polyacrylamide-gel electrophoresis and stained with Coomassie Blue as described previously (Bury, 1981). Samples are: lane 1, M_r standards; lane 2, initial extract; lane 3, post-DEAE-Sephacel; lane 4, post-Sepharose 6B; lane 5, post-Mono Q; lane 6, post-phenyl-Sepharose.

pH optimum for human multicatalytic proteinase for hydrolysis of Z-Gly-Gly-Arg-NHMec and Suc-Ala-Ala-Phe-NHMec

The pH–activity profile for human multicatalytic proteinase was performed using buffers of constant molarity or constant conductivity (Fig. 2). For Z-Gly-Gly-Arg-NHMec, optimal activity was recorded at pH 10.0 or above, whereas for Suc-Ala-Ala-Phe-NHMec optimal activity was recorded at pH 4.5. A shoulder of activity against Suc-Ala-Ala-Phe-NHMec was also recorded at pH 7.5, although activity at pH 4.5 was at least 5-fold higher. This is the first time that an acidic pH optimum has been noticed for any substrate by any species of multicatalytic proteinase. Dahlmann *et al.* (1985) showed that the rat enzyme also hydrolysed Suc-Ala-Ala-Phe-NHMec, but with a pH optimum of 7.5. Activity was not measured below pH 6.5, however, and hence the true pH optimum for this substrate and enzyme may well have been missed. Tanaka *et al.* (1986) also reported a pH optimum of 7.0 for a related substrate, glutaryl-Ala-Ala-Phe-methoxynaphthylamide, but again did not report activity below pH 6.0.

Influence of buffer composition on activity

A major consideration in the study of multicatalytic proteinase is its sensitivity to the ionic strength of buffers used during assay. Wilk & Orlowski (1983) originally reported that the enzyme from bovine pituitary was inhibited by low concentrations of KCl. This important factor is often overlooked and may well affect the apparent activity of the enzyme when proteinase inhibitors are used. I reinvestigated this using a range of buffers of different concentration and composition (Fig. 3). These results show that the enzyme is inhibited by higher concentrations of buffers, which is related to

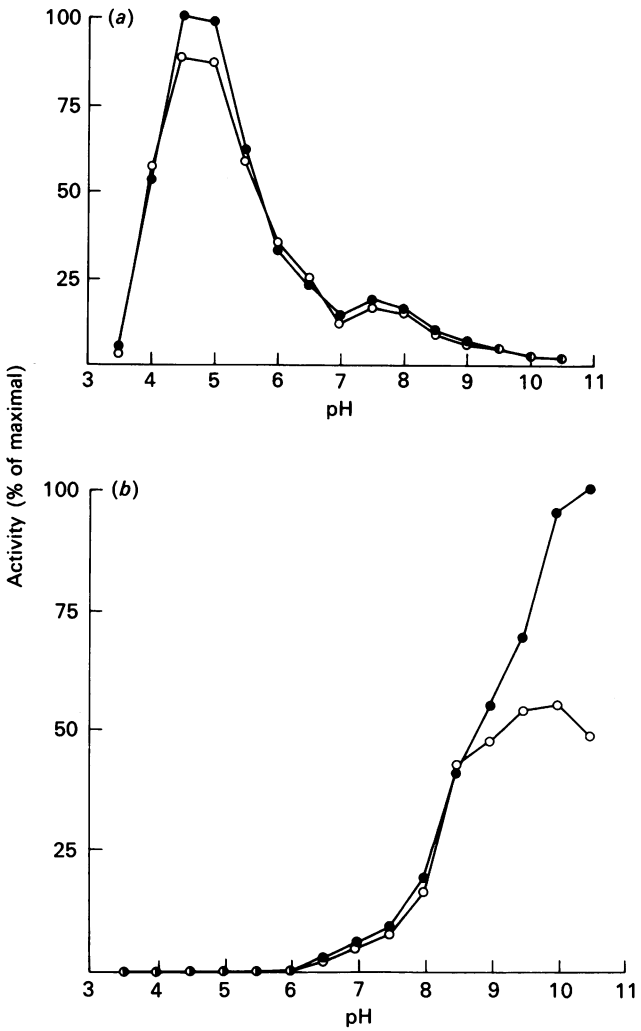

Fig. 2. pH–activity profiles for multicatalytic proteinase

A series of buffers of constant conductivity of 1.00 μS were prepared. They were sodium formate (pH 3.5–4.5), sodium acetate (pH 4.5–5.5), sodium phosphate (pH 5.5–7.5), Tris/HCl (pH 7.5–8.5) and sodium carbonate (pH 8.5–10.5). A similar series of buffers of a constant molarity of 50 mM were also prepared. The figures show activity profiles with buffers of constant conductivity (●) or constant molarity (○). Values are expressed as a percentage of maximal activity recorded for each substrate. (*a*) Activity against Suc-Ala-Ala-Phe-NHMec; (*b*) activity against Z-Gly-Gly-Arg-NHMec.

their conductivity. The importance of choice of buffer is particularly evident when measuring the activity of multicatalytic proteinase against Z-Gly-Gly-Arg-NHMec at pH 10.0. The activity in 50 mM-diethanolamine/HCl, a buffer of low conductivity, is over three times higher than in 50 mM-sodium borate buffer.

Michaelis constant determination

The K_m for hydrolysis of Suc-Ala-Ala-Phe-NHMec was determined at pH 4.5 and pH 8.5 and the K_m for hydrolysis of Z-Gly-Gly-Arg-NHMec at pH 8.5 and pH 10.0 by the method of Wilkinson (1961). The maximum concentration of substrates used was 200 μM, which proved to be well below K_m, which was therefore only

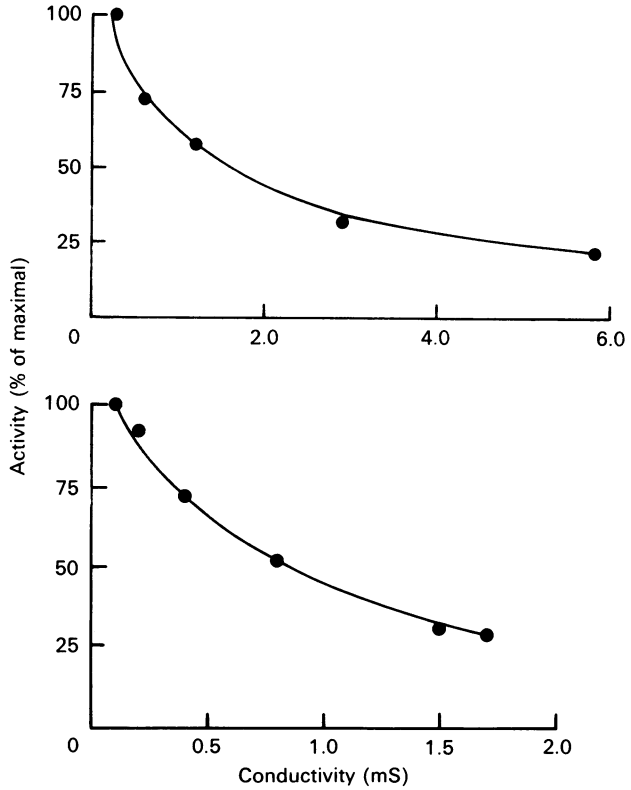

Fig. 3. Effect of buffer composition on the activity of multicatalytic proteinase

(*a*) Multicatalytic proteinase was assayed against Suc-Ala-Ala-Phe-NHMec (5 μM) in sodium acetate buffer, pH 4.5. Final buffer concentrations, of increasing conductivity, were 25, 50, 100, 250 and 500 mM. (*b*) The activity of multicatalytic proteinase against Z-Gly-Gly-Arg-NHMec was assayed in a range of buffers at pH 10.0. The buffers, of increasing conductivity, were diethanolamine/HCl (25, 50 and 100 mM), sodium carbonate (50 and 100 mM) and sodium borate (50 mM) Activities are expressed as a percentage of maximal activity and are plotted against buffer conductivity.

estimated as being greater than 500 μM for each substrate and at each pH value.

Effect of inhibitors of aspartic and metallo- proteinases

Pepstatin (2.5 μM), 1,10-phenanthroline (1 mM) and EDTA (10 mM) inhibited activity against Suc-Ala-Ala-Phe-NHMec at pH 4.5 and Z-Gly-Gly-Arg-NHMec at pH 10 by less than 10%. These results suggest that multicatalytic proteinase is neither a typical aspartic proteinase nor a typical metallo-proteinase.

Effect of peptidyl aldehydes

Peptidyl aldehydes are reversible inhibitors of serine and cysteine proteinases (Umezawa, 1982). Chymostatin inhibited activity against Suc-Ala-Ala-Phe-NHMec with a K_i of 160 ± 22 μM at pH 4.5. Leupeptin (200 μM) did not inhibit this activity. Both leupeptin and chymostatin inhibited activity against Z-Gly-Gly-Arg-NHMec at pH 10.0, behaving as very slow binding reversible inhibitors. The major difference between chymostatin and leupeptin is that the former has phenylalininal in P_1 whereas the latter has argininal in P_1 (see Powers & Harper, 1986, for complete structures). Binding of these inhibitors was so slow that determination of K_i was not possible with continuous rate assays. With such assays, when $[I] \gg K_i$, k_{on} could be determined (Baici, 1988). Using 1–10 μM-leupeptin, it was found that the rate of inactivation ($k_{obs.}$) was proportional to inhibitor concentration and k_{on} was determined as 1290 ± 125 $M^{-1} \cdot s^{-1}$. Thus the half-life of enzyme increased with decreasing inhibitor concentration such that at 10 μM the half-life was 9 min. An upper estimate of K_i was determined by incubating the enzyme with 0–200 nM inhibitor for 16 h prior to addition of substrate. The K_i was determined as 25 ± 5 nM. Even slower-binding reversible inhibition was seen in the inhibition of activity against Z-Gly-Gly-Arg-NHMec by chymostatin using 50–200 μM inhibitor. In this case k_{on} was determined as 12 ± 2 $M^{-1} \cdot s^{-1}$ and K_i as 5 ± 1.5 μM.

Such slow-binding reversible inhibition has been seen for peptidyl trifluoromethanes interacting with a cysteine proteinase, cathepsin B (Smith *et al.*, 1988), and the serine proteinases chymotrypsin and pancreatic elastase (Imperiali & Abeles, 1986). Peptidyl aldehydes are normally much faster binding reversible inhibitors, the k_{on} for leupeptin binding to cathepsin B being 180000 $M^{-1} \cdot s^{-1}$ (Baici & Gyger-Marazzi, 1982).

Activities of multicatalytic proteinase against two similar substrates exhibiting differential inhibition characteristics but at different pH values have previously been taken as an indication of more than one active site (Wilk & Orlowski, 1983). However it is noteworthy that the pH optimum for activity against Z-Gly-Gly-Arg-NHMec is above pH 10.0. High pH values would neutralize the charge of the arginine side chain (pK 12.5) in both the substrate and the inhibitor, leupeptin. The electrostatic environment in a single active site is likely to be quite different at pH 4.5 than at pH 10.0.

In order to obtain information on whether multicatalytic proteinase really has two different catalytic sites for these substrates, I performed experiments at pH 8.5. Although activity against both substrates was considerably reduced, activity could be measured by using 100 μM substrate. Activity against Z-Gly-Gly-Arg-NHMec was inhibited by leupeptin and chymostatin with similar values of k_{on} and K_i as seen at pH 10. Activity against Suc-Ala-Ala-Phe-NHMec was inhibited by chymostatin (K_i 150 ± 20 μM) and not inhibited by leupeptin (200 μM). This clearly shows that the two activities are distinct. When the enzyme was assayed in the presence of both substrates, activity was additive and inhibition by leupeptin and chymostatin was consistent with independent inhibition of the Z-Gly-Gly-Arg-NHMec and Suc-Ala-Ala-Phe-NHMec hydrolysing activities respectively.

Effect of peptidyl epoxides, diazomethanes and chloromethanes

Multicatalytic proteinase is reported to be either a cysteine proteinase or a serine proteinase. Serine proteinases can usually be inhibited by peptidyl chloromethanes which react covalently with active site residues (Powers, 1977). Cysteine proteinases can be inhibited by peptidyl chloromethanes or diazomethanes and peptidyl epoxides (Rich, 1986). Inhibition by such compounds is dependent upon concentration and incubation time, and provided that the reagent is in excess complete inhibition should be obtained.

Multicatalytic proteinase was not inhibited by E-64, a

Table 2. Effect of peptidyl diazomethanes and chloromethanes and E-64 on the activity of multicatalytic proteinase

Values shown are the means of at least three separate determinations and are based on activity inhibited after incubation of compounds with enzyme in the presence of substrate for 30 min. No significant time-dependent inhibition was noted for any inhibitor.

Inhibitor	Concentration (μM)	Inhibition (%) of activity against: Suc-Ala-Ala-Phe-NHMec	Z-Gly-Gly-Arg-NHMec
Pro-Phe-Arg-CH_2Cl	100	10	7
Tos-Lys-CH_2Cl	100	5	5
Z-Leu-Lys-CHN_2	100	16	20
Z-Gly-Leu-Phe-CH_2Cl	100	20	7
Z-Phe-Phe-CHN_2	100	12	8
Z-Leu-hPhe-CHN_2	100	5	5
Z-Phe-Ala-CHN_2	100	33	12
Z-Tyr-Ala-CHN_2	100	15	6
E-64	100	5	5

compound isolated from *Aspergillus japonicus* which inhibits a variety of mammalian cysteine proteinases (Barrett *et al.*, 1982). However, lack of inhibition by E-64 does not necessarily indicate that a given proteinase is not a cysteine proteinase, as not all cysteine proteinases are inhibited by this compound. Inhibition by such compounds is dependent upon binding of the side chains of E-64 prior to covalent binding of the epoxide to the active-site cysteine, and thus specificity of an enzyme will determine its reactivity towards E-64.

Peptidyl chloromethanes and diazomethanes are active-site-directed inhibitors of serine and cysteine proteinases (Powers & Harper, 1986; Rich, 1986). None of the inhibitors used irreversibly inhibited multicatalytic proteinase in a time-dependent manner using either substrate, and only slight non-specific or possibly weak reversible inhibition could be detected with high concentrations of inhibitors.

Lack of inhibition by any of these active-site-directed irreversible inhibitors of cysteine and serine proteinases suggests that multicatalytic proteinase is not a typical cysteine or serine proteinase.

Effect of thiol reagents

Iodoacetamide inhibited multicatalytic proteinase activity against Z-Gly-Gly-Arg-NHMec at pH 10.0. Inhibition was dependent upon inhibitor concentration and incubation time, and the rate of inactivation was determined to be 0.75 $M^{-1} \cdot s^{-1}$. Inhibition could not be demonstrated at pH 8.5 or lower, and iodoacetic acid (1 mM) was not inhibitory at all in the pH range 4.5–10 for activity against either Suc-Ala-Ala-Phe-NHMec or Z-Gly-Gly-Arg-NHMec. These very slow inhibition characteristics are not consistent with the inhibition of cysteine proteinases; papain reacts with iodoacetamide at pH 10.35 at a rate of 976 $M^{-1} \cdot s^{-1}$, and with iodoacetate at pH 6.0 at a rate of 1100 $M^{-1} \cdot s^{-1}$ (Polgar & Csoma, 1987), whereas the rates of reaction of iodoacetamide and iodoacetate with simple thiols such as glutathione are much slower [27 and 2.8 $M^{-1} \cdot s^{-1}$ respectively (Halasz & Polgar, 1977)]. This suggests that the essential thiols of the enzyme are not particularly reactive and may be partially masked. When ^{14}C-labelled iodoacetamide was used to inhibit the enzyme, subsequent fluorography of SDS/polyacrylamide gels showed that the inhibitor labelled all of the protein bands, showing no preferential labelling of possible specific catalytic subunits (results not shown).

Inhibition by *N*-ethylmaleimide was also very slow. The rate of inactivation of hydrolysis of Suc-Ala-Ala-Phe-NHMec and Z-Gly-Gly-Arg-NHMec at pH 8.5 was only 1.4 ± 0.04 and 1.6 ± 0.04 $M^{-1} \cdot s^{-1}$, respectively. $HgCl_2$ (100 μM) completely inhibited enzymic activity against Suc-Ala-Ala-Phe-NHMec and Z-Gly-Gly-Arg-NHMec at pH 4.5 and 10.0, respectively. Inhibition was readily reversible by addition of dithiothreitol.

Effect of di-isopropyl fluorophosphate

Di-isopropyl fluorophosphate binds irreversibly to the active site of most serine proteinases and inhibition is both time- and concentration-dependent (Powers & Harper, 1986). Activity against Z-Gly-Gly-Arg-NHMec at pH 10 was instantaneously reduced by addition of di-isopropyl fluorophosphate to a final concentration of 25 mM, but this was found to be due to reduction of the pH to 7.5 in the weak assay buffer. When the pH was adjusted to 10, the enzyme activity was stable for at least 30 min in the presence of 25 mM-di-isopropyl fluorophosphate. Final confirmation that multicatalytic proteinase does not react with di-isopropyl fluorophosphate was determined when the enzyme was treated with 12.5 mM-[^3H]di-isopropyl fluorophosphate at pH 4.5, 7.5 and 10.0 for 1 h at 37 °C followed by SDS/polyacrylamide-gel electrophoresis and fluorography. No reaction with the inhibitor could be detected. As a positive control, it was found that the heavy chain of chymotrypsin was labelled at pH 8.0 (results not shown).

These results demonstrate that the multicatalytic proteinase is not a serine proteinase that can be inhibited by di-isopropyl fluorophosphate. This may explain the variability of labelling of a range of species variants of multicatalytic proteinase by ^3H-labelled di-isopropyl fluorophosphate seen by Tanaka *et al.* (1986). They found that several bands of the rat enzyme were heavily labelled whereas only one band of human multicatalytic proteinase was only weakly labelled.

Effect of dichloroisocoumarin

Dichloroisocoumarin has been developed as an inhibitor of serine proteinases (Harper *et al.*, 1985). It has

been reported not to inhibit a cysteine proteinase, papain, although under the conditions used, i.e. pH 8.2 and 5 mM-cysteine, the inhibitor is rapidly inactivated. I therefore considered that a reinvestigation of the inhibition of cysteine proteinases was necessary. The stability of the inhibitor under incubation conditions used was determined by measuring the reduction in absorption at 325 nm as described by Harper *et al.* (1985). The times taken for inactivation of 50% of the inhibitor at pH 10, 8.5 and 4.5 were <5 s, 6 min and >2 h, respectively. The stability of the inhibitor was greatly reduced in the presence of 1 mM-dithiothreitol or 5 mM-cysteine, such that the times taken for inactivation of 50% of the inhibitor at pH 8.5 and 4.5 were reduced to 10 s and 6 min, respectively.

It was not possible to obtain a linear rate of substrate hydrolysis by the cysteine proteinases papain, cathepsin B, cathepsin L or cathepsin H using less than 1 mM-dithiothreitol in continuous rate assays, but even when 100 μM-dichloroisocoumarin was added to enzyme in the presence of 1 mM-dithiothreitol at pH 4.5, the activity of none of the enzymes was lost during the first 10 min of incubation. This suggests that although dichloroisocoumarin can be hydrolysed by free thiols, it is not particularly reactive with the active site thiols of cysteine proteinases.

Due to the instability of the inhibitor, it was not possible to measure the inhibition of Z-Gly-Gly-Arg-NHMec-hydrolysing activity of multicatalytic proteinase by dichloroisocoumarin. Activity against Suc-Ala-Ala-Phe-NHMec at pH 4.5 was inhibited by dichloroisocoumarin. The inhibition characteristics were typical for irreversible active-site-directed inhibitors. Rates of inactivation were determined using 10–100 μM inhibitor. The apparent second-order rate constant for inactivation, $k_{obs.}/[I]$, increased with decreasing concentrations of inhibitor, indicating that the condition $[I] \ll K_i$ was not met. Therefore the individual rate constants, k_{+2} and K_i, were determined as described previously (Crawford *et al.*, 1988). K_i was determined to be 63 ± 18 μM and k_{+2} to be 0.016 ± 0.002 s^{-1} and hence k_{+2}/K_i as 250 $M^{-1} \cdot s^{-1}$.

Some 80% of activity against Z-Gly-Gly-Arg-NHMec at pH 10 was lost when the enzyme was preincubated with 100 μM-dichloroisocoumarin for 30 min at pH 4.5, indicating that this inhibitor also reacts with the active site responsible for hydrolysis of this substrate.

Dichloroisocoumarin was the only active-site-directed irreversible inhibitor of proteinases that was found to have any effect on multicatalytic proteinase. I have confirmed that this compound is not particularly reactive with cysteine proteinases of the papain superfamily, although it does react with free thiols. The rate of inactivation of the Suc-Ala-Ala-Phe-NHMec-hydrolysing activity of multicatalytic proteinase by this compound was slower than the rate of inactivation of leukocyte elastase, but faster than the rate of inactivation of other serine proteinases including cathepsin G, plasmin and trypsin (Harper *et al.*, 1985).

It therefore appears that multicatalytic proteinase is an atypical serine proteinase that is not inhibited by diisopropyl fluorophosphate, peptidyl diazomethanes or peptidyl chloromethanes. It also has thiols that are not particularly reactive, but must be free in order for the enzyme to exhibit activity. The slow-binding characteristics of inhibition of activity against Z-Gly-Gly-Arg-NHMec by peptidyl aldehydes suggests that access to this active site of the enzyme is restricted. This might explain why the purified enzyme appears to be latent with regard to hydrolysis of more bulky protein substrates (Tanaka *et al.*, 1986).

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