Reaction of proteasomes with peptidylchloromethanes and peptidyldiazomethanes

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The multicatalytic endopeptidase complex (proteasome) has multiple distinct peptidase activities. These activities have often been referred to as 'chymotrypsin-like', 'trypsin-like' and 'peptidylglutamyl-peptide hydrolase' activities according to the type of residue in the P1 position, although it is now clear that mammalian proteasomes have at least five distinct catalytic sites. In the present study, potential affinity-labelling reagents (peptidylchloromethanes, peptidyldiazomethanes, a peptidyl-fluoromethane and peptidylsulphonium salts) containing hydrophobic, basic or acidic amino acid residues in the P1 position have been tested for inhibition of the different activities of the rat liver proteinase complex. The results show that individual peptidase activities of proteasomes can be inhibited by a variety of peptidylchloromethanes and peptidyldiazomethanes. Although the rate of inactivation of proteasomes by even the most effective peptidylchloromethanes and peptidyldiazomethanes are often quite slow ($k_{\text{obs.}}/[I]$ in the range 0.1–10 M⁻¹·s⁻¹) compared with the reaction of similar compounds with some other protein-

ases, the results provide useful information concerning the specificity of the distinct catalytic centres of proteasomes, and some selective affinity-labelling reagents have been identified. Tyr-Gly-Arg-chloromethane was found to be a useful inhibitor of trypsin-like activity. Inhibition of the other peptidase activities was often incomplete, even after repeated addition of inhibitor, and it proved to be difficult to predict the effect of different reagents. For example, Cbz-Tyr-Ala-Glu-chloromethane was found to inhibit 'chymotrypsin-like' activity (assayed with Ala-Ala-Phe-7-amino-4-methylcoumarin or succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin), while the best inhibitors of 'peptidylglutamyl-peptide hydrolase' activities (assayed with benzyloxycarbonyl-Leu-Leu-Glu β -naphthylamide) were peptidyldiazomethanes containing hydrophobic amino acid residues. These results suggest that the original nomenclature of proteasome activities is misleading, because the residue in the P1 position is not the only determinant of specificity.

INTRODUCTION

The multicatalytic proteinase complex or proteasome (multicatalytic endopeptidase complex, EC 3.4.99.46) is a high-molecular-mass nonlysosomal proteinase of eukaryotic cells (Orlowski, 1990; Tanaka et al., 1992; Rivett, 1993). The proteinase can cleave peptide bonds on the carboxy side of hydrophobic, basic or acidic amino acid residues, and these activities have been referred to as 'chymotrypsin-like', 'trypsin-like' and 'peptidylglutamyl-peptide hydrolase' activities respectively (Orlowski, 1990). Mammalian proteasomes are now believed to contain at least five distinct catalytic components (Djaballah et al., 1992), the activities of which can be distinguished by assessing the effects of activators and inhibitors of proteolytic activity (Orlowski and Michaud, 1989; Rivett, 1989b; Cardozo et al., 1992; Djaballah et al., 1992). For the rat liver enzyme, the catalytic components include one catalysing trypsin-like activity, two catalysing the hydrolysis of substrates for chymotrypsin-like activity, and two catalysing the so-called 'peptidylglutamyl-peptide hydrolase' activity. We have referred to the latter two components as LLE1 and LLE2 (Djaballah and Rivett, 1992) because both are able to hydrolyse the synthetic substrate benzyloxycarbonyl (Cbz)-Leu-Leu-Glu- β -naphthylamide (Nap). They can be distinguished easily because LLE2 is inhibited by the serine-proteinase inhibitor, 3,4-dichloroisocoumarin, while LLE1 is unaffected. The other activities can be assayed individually using an appropriate substrate. Rather

little is known about the precise specificity of any of the catalytic components of the complex.

For several years the assignment of proteasomes into the class of serine or cysteine proteinases was a controversial issue because they were often found to be largely unaffected by the serine-protease inhibitor di-isopropyl fluorophosphate, but were sensitive to inhibition by thiol-reactive reagents (Rivett, 1989a). However, the demonstration that at least some activities of proteasomes are effectively inhibited by the serine-protease inhibitors 3,4-dichloroisocoumarin (Orlowski and Michaud, 1989; Mason, 1990; Djaballah et al., 1992) and 4-(2-aminoethyl)benzenesulphonic acid (Djaballah et al., 1992) has favoured the former classification. The lack of sequence similarity to other known proteases (Tanaka et al., 1992; Rivett, 1993) suggests that proteasomes belong to a novel family of peptidases.

The purpose of the present study was to learn more about the specificity and reactivity of the different catalytic centres of the complex, and also to identify suitable reagents for the identification of catalytic components and residues responsible for these activities. Peptidylchloromethanes, peptidyldiazomethanes, peptidylsulphonium salts and peptidylfluoromethanes have been used successfully as affinity-labelling reagents for many serine and/or cysteine proteinases (Shaw, 1984, 1990). In the present study, reagents designed to inhibit proteinases having a preference for hydrophobic, basic or acidic amino acid residues in the P1 position have been tested for inhibition of proteasome activities. It seems, from kinetic studies with synthetic fluorogenic

Abbreviations used: Bz, benzoyl; Bzl, benzyl; Boc, *N*-t-butoxycarbonyl; Cbz, benzyloxycarbonyl; -CH₂Cl, chloromethane, -CH₂F, fluoromethane; -CHN₂, diazomethane; -CH₂S⁺(CH₃)₂, sulphonium salt; $k_{\text{obs.}}$, observed pseudo-first-order rate constant; NH-Mec, 7-amino-4-methylcoumarin; Nap, β -naphthylamide; Suc, succinyl; OSu, succinyl ester; O-t-Bu, O-t-butyl ester.

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peptide substrates for chymotrypsin-like and trypsin-like activities of proteasomes, that the enzyme may have a preference for a tripeptide or tetrapeptide rather than only a dipeptide or single amino acid attached to the leaving group (McGuire and DeMartino, 1986; Rivett, 1989b). Therefore it was of particular interest to investigate the effects of potential affinity-labelling reagents containing a variety of peptidyl portions rather than single amino acid derivatives.

METHODS

Materials

Rats (Wistar) were obtained from the University of Leicester Biomedical services unit. *N*-t-Butoxycarbonyl (Boc)-Leu-Ser-Thr-Arg-7-amino-4-methylcoumarin (NH-Mec) was purchased from the Peptide Institute, Osaka, Japan. Ala-Ala-Phe-NH-Mec, succinyl (Suc)-Leu-Leu-Val-Tyr-NH-Mec and benzyloxycarbonyl (Cbz)-Leu-Leu-Glu- β -naphthylamide (Nap) were from Sigma Chemical Co.

Peptidylchloromethanes were synthesized as described by Kettner and Shaw (1981), except those containing tyrosine, which were prepared as described by Rauber et al. (1988). Cbz-Tyr-Ala-Glu-chloromethane (CH_2Cl) and Cbz-Leu-Leu-Glu- CH_2Cl were synthesized as described below.

Cbz-Leu-Tyr fluoromethane (CH_2F) was obtained as described by Angliker et al. (1989). The peptidylsulphonium salts containing arginine were described by Zumbunn et al. (1988), whereas the synthesis of the derivative of Cbz-Leu-Tyr has not been published yet (but details can be obtained from H. A. or E. S. on request). Peptidyl diazomethanes Cbz-Phe-Ala-diazomethane (CHN_2) and Cbz-Ala-Phe-Ala- CHN_2 were synthesized as described by Watanabe et al. (1979), and Cbz-Leu-Trp- CHN_2 , Cbz-Phe-benzylserine [Ser(Bzl)]-Leu-Tyr- CHN_2 and Boc-Val-Lys(Cbz)-Leu-Tyr- CHN_2 were prepared as described by Crawford et al. (1988); Cbz-Ala-Ala-Leu- CHN_2 as described by Green and Shaw (1981); Cbz-Phe-Gly-Tyr- CHN_2 as described below.

Synthesis of Cbz-Phe-Gly-Tyr- CHN_2

Cbz-Phe-GlyOH was activated as the hydroxysuccinimide ester with dicyclohexyl carbodi-imide in the standard fashion and allowed to react with tyrosine in aq. 50% dioxan with added NaHCO_3 to provide Cbz-Phe-Gly-Tyr. The blocked tripeptide was converted into the mixed anhydride and added to ethereal diazomethane to form the peptidyl diazomethane. The neutral product was chromatographed on Kieselgel in chloroform containing 2% methanol and crystallized from ethyl acetate to provide material of m.p. 117–118 °C. Analysis: calculated for $\text{C}_{29}\text{H}_{29}\text{N}_5\text{O}_6$ (543.6): C, 64.02; H, 5.38; N, 12.88; found: C, 63.83; H, 5.69; N, 12.56.

The remaining diazomethanes which have not been described previously were obtained by a similar procedure.

Synthesis of Cbz-Tyr-Ala-Glu- CH_2Cl and Cbz-Leu-Leu-Glu- CH_2Cl

Glutamic acid was incorporated as the γ -*O*-t-butyl ester (*O*-t-Bu), which was maintained through the tripeptide diazomethane stage, but finally deblocked with HCl during the conversion into the chloromethane. Thus Cbz-Tyr-Ala-succinyl ester (OSu) + $\text{H}_2\text{N-Glu}(\text{O-t-Bu})\text{OH}$ provided Cbz-Tyr-Ala-Glu(*O*-t-Bu)OH, from which Cbz-Tyr-Ala-Glu(*O*-t-Bu)- CHN_2 , m.p. 115–116 °C, was obtained. Analysis: calculated for $\text{C}_{30}\text{H}_{37}\text{N}_5\text{O}_8$ (595.65): C,

60.49; H, 6.26; N, 11.76; found: C, 59.90; H, 6.41; N, 11.42. Cbz-Tyr-Ala-Glu- CH_2Cl was formed from the blocked diazomethane by treatment with HCl gas in ethyl acetate at room temperature. After 45 min the mixture was evaporated to dryness with a strong stream of N_2 and the residue provided crystals from tetrahydrofuran of m.p. 162–164 °C. Calculated for $\text{C}_{26}\text{H}_{30}\text{N}_3\text{O}_7\text{Cl}$: Cl, 6.47. Found: Cl 6.24.

Cbz-Leu-Leu-Glu(*O*-t-Bu)OH was synthesized from Cbz-Leu-Leu as in the preceding example and converted into the diazomethane in the standard fashion providing crystals, m.p. 160–161 °C. Analysis: calculated for $\text{C}_{30}\text{H}_{45}\text{N}_5\text{O}_7$: C, 61.31; H, 7.71; N, 11.92; found: C, 61.26; H, 7.70; N, 11.80. A solution of the diazomethane in 2 M-HCl in ethyl acetate was kept at room temperature for 3 h and then evaporated to dryness. The residue crystallized on standing and was thinned with ethyl acetate and hexane for isolation of crystals. Mass: calculated for $\text{C}_{26}\text{H}_{38}\text{N}_3\text{O}_7\text{Cl}$: *m/z*, 539; found: *m/z*, 539.

Proteinase purification and assays

Proteasomes were purified from fresh or frozen rat livers (Rivett and Sweeney, 1991). Protein determinations were carried out by the method of Bradford (1976), with BSA as standard. This method gives a very good approximation to actual proteasome concentration (Savory and Rivett, 1993). The proteinase was assayed as described previously in 50 mM Hepes/KOH buffer, pH 7.5 (Rivett, 1989b; Djaballah et al., 1992) using the following substrates: 40 μM Boc-Leu-Ser-Thr-Arg-NH-Mec for trypsin-like activity, 40 μM Ala-Ala-Phe-NH-Mec and Suc-Leu-Leu-Val-Tyr-NH-Mec for the chymotrypsin-like activities, and 0.1 mM and 0.4 mM Cbz-Leu-Leu-Glu-Nap for peptidyl-glutamyl-peptide hydrolase activities. At 0.1 mM Cbz-Leu-Leu-Glu-Nap the activity assayed is predominantly that of the LLE1 site, while at 0.4 mM, LLE2 is the major component (Djaballah and Rivett, 1992).

Inhibitor studies

For initial studies of the effects of inhibitors, proteinase (20–40 $\mu\text{g/ml}$) was incubated with 0.1 mM inhibitor at 25 °C in 50 mM Hepes buffer, pH 7.0 or 7.5. Duplicate aliquots were removed after 0, 1 and 2 h, diluted at least 10-fold into 50 mM Hepes buffer, pH 7.5, containing the appropriate substrate for assays of proteinase activity (10–20 min at 37 °C). The reversibility of the inhibition was tested by assaying activity following overnight dialysis to remove excess inhibitor. The rate of inactivation was calculated for the most effective inhibitors under pseudo-first-order conditions (i.e. $[\text{I}] \gg [\text{E}]$). Apparent rate constants (k_{obs}) were determined from plots of log(activity remaining) against preincubation time. Apparent second-order rate constants for inactivation were calculated as $k_{\text{obs}}/[\text{I}]$.

The stability of peptidyl diazomethane inhibitors was tested by measuring A_{277} before and after incubation with proteinase (Green and Shaw, 1981). Cleavage of the peptidyl portion of inhibitors was tested in some cases by reverse-phase h.p.l.c. using a Vydac C_{18} column with a linear gradient of 0–70% acetonitrile in 0.05% trifluoroacetic acid.

RESULTS

Several peptidylchloromethanes with an arginine residue in the P1 position were tested for inhibition of Boc-Leu-Ser-Thr-Arg-NH-Mec hydrolysis (Table 1). Glu-Gly-Arg- CH_2Cl and Tyr-Gly-Arg- CH_2Cl both inhibited the trypsin-like activity in a time-dependent and irreversible manner, whereas several other com-

Table 1 Effect of affinity labels with a basic residue in P1 on trypsin-like activity of rat liver proteasomes

Activities, assayed with Boc-Leu-Ser-Thr-Arg-NH-Mec after 1 h preincubation in 50 mM Hepes/KOH buffer, pH 7.5, as described in the Materials and methods section, are expressed as a percentage of the activity of proteinase assayed immediately after addition of affinity labels. The enzyme retained more than 95% of its activity during control incubations which contained no inhibitor. The results given are averages for at least two separate experiments. Ac is acetyl.

Reagent	Concn. (mM)	Activity (% of value at t_0)
Peptidylchloromethanes		
Ac-Gly-Gly-Arg-CH ₂ Cl	0.1	123
Gln-Gly-Arg-CH ₂ Cl	0.1	123
Glu-Gly-Arg-CH ₂ Cl	1	39
	0.1	95
Ile-Gly-Gly-Arg-CH ₂ Cl	0.1	100
Tyr-Gly-Arg-CH ₂ Cl	0.1	24
	0.02	62
Tyr-Phe-Arg-CH ₂ Cl	0.1	123
Peptidylsulphonium salts		
Bz-Phe-Arg-CH ₂ S ⁺ (CH ₃) ₂	0.1	134
Ala-Arg-Arg-CH ₂ S ⁺ (CH ₃) ₂	0.1	108
Peptidyl diazomethane		
Boc-Val-Leu-Gly-Lys-CHN ₂	0.1	76

pounds had little effect. Two peptidylsulphonium salts did not inhibit. Boc-Val-Leu-Gly-Lys-CHN₂ reacted only slowly, and much of the inhibition observed (Table 1) was due to nonspecific effects rather than irreversible modification. This peptidyl-

diazomethane inhibitor was not a substrate for the proteinase and there was no loss of the diazo group during the incubations. In some cases, the trypsin-like activity was found to increase consistently by 20–30% following a 1 h incubation with a peptidylchloromethane or peptidylsulphonium salt and was higher than that of control preparations (Table 1).

The effects of various potential affinity-labelling reagents on chymotrypsin-like and peptidylglutamyl-peptide hydrolase activities are shown in Table 2. Several inhibitors with hydrophobic residues in the P1 position, which might be expected to inhibit chymotrypsin-like activities assayed with Ala-Ala-Phe-NH-Mec and Suc-Leu-Leu-Val-Tyr-NH-Mec, were also found to inhibit peptidylglutamyl-peptide hydrolase activities assayed with Cbz-Leu-Leu-Glu-Nap (Table 2). Several of these affinity-labelling reagents, especially the diazomethanes, did inhibit proteasome activities in an irreversible, time-dependent, manner. There was no cleavage of the inhibitors tested, but in some cases a direct loss of diazo groups was observed (e.g. for Cbz-Phe-Gly-Tyr-CHN₂; approx. 50% in 3 h).

Cbz-Phe-Gly-Tyr-CHN₂ was found to be an effective inhibitor of chymotrypsin-like activity assayed with either Ala-Ala-Phe-NH-Mec or Suc-Leu-Leu-Val-Tyr-NH-Mec, but it was impossible to achieve full inhibition even after repeated additions of inhibitor or long incubation times. Cbz-Leu-Leu-Tyr-CHN₂ inhibited both chymotrypsin-like and peptidylglutamyl-peptide hydrolase activities, while, surprisingly, the chloromethane derivative of Cbz-Tyr-Ala-Glu did not affect the rate of hydrolysis of Cbz-Leu-Leu-Glu-Nap, but did inhibit the hydrolysis of Ala-Ala-Phe-NH-Mec and Suc-Leu-Leu-Val-Tyr-NH-Mec (Table 2). Cbz-Leu-Leu-Glu-CH₂Cl, which might be expected to selectively inhibit LLE1 and LLE2 activities, also inhibited chymotrypsin-

Table 2 Effect of inhibitors with hydrophobic or acidic P1 residue on different proteasome activities

All inhibitors were tested at a concentration of 0.1 mM. Activities given as percent of control where control incubations contained no inhibitor. Preincubation was for 1 h at 25 °C, pH 7.0, except where indicated by an asterisk, where preincubation was for 30 min at 25 °C. The enzyme retained more than 95% of its activity during control incubations containing no inhibitor. The results given are averages for at least two separate experiments. Abbreviations: nd, not determined; AAF-NH-Mec, Ala-Ala-Phe-NH-Mec; LLVY-NH-Mec, Suc-Leu-Leu-Val-Tyr-NH-Mec; LLE-Nap, Cbz-Leu-Leu-Glu-Nap.

Inhibitor	Substrate ...	Activity (% of control)			
		AAF-NH-Mec	LLVY-NH-Mec	0.1 LLE-Nap	0.4 LLE-Nap
Peptidylidiazomethanes					
	Cbz-Ala-Phe-CHN ₂	102	104	nd	nd
	Cbz-Cit-Phe-CHN ₂	104	111	nd	nd
	Cbz-Leu-Trp-CHN ₂	108	76	89	46
	Cbz-Phe-Ala-CHN ₂	103	126	nd	nd
	Cbz-Ala-Phe-Ala-CHN ₂	92	52	nd	nd
	Cbz-Ala-Ala-Leu-CHN ₂	105	64	82	68
	Cbz-Ala-Ala-Val-CHN ₂	99	85	84	84
	Cbz-Phe-Gly-Tyr-CHN ₂	41	41	94	84
	Cbz-Leu-Leu-Tyr-CHN ₂	43	27	37	43
	Boc-His-Leu-Leu-Tyr-CHN ₂	82	nd	nd	nd
	Cbz-Phe-Ser(Bzl)-Leu-Tyr-CHN ₂	84	nd	nd	nd
	Boc-Val-Lys(Cbz)-Leu-Tyr-CHN ₂	98	nd	nd	nd
Peptidylchloromethanes:					
	Ala-Ala-Phe-CH ₂ Cl	97	45	89	96
	Cbz-Gly-Gly-Phe-CH ₂ Cl	81	nd	nd	nd
	Cbz-Tyr-Ala-Glu-CH ₂ Cl ^a	64	48	96	106
	Leu-Leu-Glu-CH ₂ Cl	98	65	81	96
	Cbz-Leu-Leu-Glu-CH ₂ Cl	81	49	79	96
Peptidylsulphonium salt:					
	Cbz-Leu-Tyr-CH ₂ S ⁺ (CH ₃) ₂	97	nd	nd	nd
Peptidylfluoromethane:					
	Cbz-Leu-Tyr-CH ₂ F	101	nd	nd	nd

Table 3 Rate constants for inactivation of proteasome activities

Values are the average of three determinations.

Substrate/inhibitor	Concn. (mM)	$t_{1/2}$ (min)	$k_{\text{obs.}}$ (s^{-1})	$k_{\text{obs.}}/[I]$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Boc-Leu-Ser-Thr-Arg-NH-Mec				
Thr-Gly-Arg-CH ₂ Cl	0.1	15	7.7×10^{-4}	7.7
Boc-Val-Leu-Gly-Lys-CHN ₂	0.1	169	0.68×10^{-4}	0.68
Ala-Ala-Phe-NH-Mec				
Cbz-Phe-Gly-Tyr-CHN ₂	0.1	113	1.0×10^{-4}	1.0
Cbz-Tyr-Ala-Glu-CH ₂ Cl	0.1	225	0.5×10^{-4}	0.51
Suc-Leu-Leu-Val-Tyr-NH-Mec				
Cbz-Phe-Gly-Tyr-CH ₂ Cl*	0.005	< 5	$> 2.31 \times 10^{-3}$	> 460
Cbz-Phe-Gly-Tyr-CHN ₂	0.1	12.8	9.0×10^{-4}	9.0
Cbz-Tyr-Ala-Glu-CH ₂ Cl*	0.1	40.0	2.9×10^{-4}	2.9
Cbz-Leu-Leu-Glu-NA				
0.1 mM				
Cbz-Leu-Leu-Tyr-CHN ₂	0.1	40.8	2.8×10^{-4}	2.8
Cbz-Leu-Trp-CHN ₂	0.1	56.7	2.0×10^{-4}	2.0
0.4 mM				
Cbz-Leu-Leu-Tyr-CHN ₂	0.1	50.7	2.3×10^{-4}	2.3
Cbz-Leu-Trp-CHN ₂	0.1	115	1.0×10^{-4}	1.0

* Partial inhibition; see Figure 1.

Figure 1 Inhibition of activity assayed with Suc-Leu-Leu-Val-Tyr-NH-Mec by peptidylchloromethanesAssays were carried out as described in the Materials and methods section. ○, 5 μM Cbz-Phe-Gly-Tyr-CH₂Cl; □, 20 μM Ala-Ala-Phe-CH₂Cl; △, 100 μM Cbz-Tyr-Ala-Glu-CH₂Cl.

like activities. The most effective inhibitors of peptidylglutamyl-peptide hydrolase activities were found to be those with hydrophobic residues in the P1 and P2 positions (Table 2).

The apparent second-order rate constants for inactivation of proteasome activities by some of the most effective irreversible inhibitors are shown in Table 3. With Suc-Leu-Leu-Val-Tyr-NH-Mec as substrate, initial inactivation with several peptidylchloromethane inhibitors was very rapid (Figure 1), but no more than 50–70% of the activity could be inhibited, even after further additions of the inhibitor. With the diazomethane derivative of Cbz-Phe-Gly-Tyr, the initial rate of inactivation was high, but again only partial inhibition was achieved. Where there was rapid but partial inactivation, the inhibition was also found to be time-dependent (as illustrated in Figure 1) and mostly, if not entirely, irreversible. However, some reversible and non-specific inhibition was observed with several of the reagents. This may be due to the high concentrations of reagents used, and this has been noted previously for the human liver proteinase (Mason,

1990). The reagents were not routinely tested at concentrations higher than 0.1 mM because of non-specific inhibition of several of the activities.

DISCUSSION

Chloromethanes are useful reagents for the alkylation of the catalytic histidine or serine residues of serine proteinases (Shaw, 1984). Diazomethanes were initially believed to inhibit cysteine, but not serine, proteinases (Leary et al., 1977; Shaw, 1984, 1990). However, later studies showed that the serine proteinase plasma kallikrein can be inhibited by peptidyl-diazomethanes (Zumbrunn et al., 1988), as can prolyl endopeptidase (Stone et al., 1992). Chymotrypsin, on the other hand, is not inactivated by Cbz-Ala-Phe-CHN₂, but catalyses the disappearance of diazo groups, which can be monitored spectrophotometrically (Watanabe et al., 1979).

The peptidase activities of purified rat liver proteasomes can be inhibited by a variety of peptidylchloromethanes and peptidyl-diazomethanes in a time-dependent, irreversible, manner. The fact that several chloromethanes and diazomethanes were ineffective demonstrates that the choice of peptidyl portion of the inhibitor is important and explains the negative results of other studies of mammalian proteasomes in which only a few reagents were tested (Dahlmann et al., 1985; Mason, 1990; Cardozo et al., 1992). It is very difficult to predict which reagents will be effective on the different peptidase sites. For example, the lack of inhibition of Ala-Ala-Phe-NH-Mec hydrolysis by Ala-Ala-Phe-CH₂Cl was particularly surprising.

Irreversible inhibition of proteasome activities by peptidylchloromethanes and peptidyl-diazomethanes is consistent with the notion that the complex is an atypical serine proteinase (Mason, 1990; Orlowski et al., 1991; Djaballah et al., 1992). The rates of inactivation with many of the reagents tested were low compared with those for some other serine proteinases, and in many cases it was not possible to achieve complete inhibition of an activity, even after repeated additions of inhibitor. Such observations could be explained by a lack of reactivity of catalytic residues in some catalytic centres with these affinity-labelling

reagents, as found for a number of serine-proteinase inhibitors (Djaballah et al., 1992) or simply reflect a poor choice of peptidyl portions of the inhibitor.

Chymotrypsin-like activities of proteasomes, assayed with Ala-Ala-Phe-NH-Mec or Suc-Leu-Leu-Val-Tyr-NH-Mec, were inhibited by peptidyl diazomethanes containing at least three amino acids, including some hydrophobic residues. In general, the dipeptidyl derivatives tested were ineffective, and this probably reflects an extended substrate-binding region at catalytic centres for chymotrypsin-like activity. Cbz-Phe-Gly-Tyr-CHN₂ was the best inhibitor of activity assayed with Ala-Ala-Phe-NH-Mec, whereas several diazomethanes and chloromethanes were effective inhibitors of only 50–70% of activity assayed with Suc-Leu-Leu-Val-Tyr-NH-Mec. It seems likely from these results that there are at least two types of catalytic sites able to hydrolyse Suc-Leu-Leu-Val-Tyr-NH-Mec, only one of which is inhibited by Ala-Ala-Phe-CH₂Cl or Cbz-Leu-Leu-Tyr-CHN₂. Inhibition of both of the chymotrypsin-like activities by the peptidylchloromethane Cbz-Tyr-Ala-Glu-CH₂Cl suggests that a hydrophobic residue in the P1 position is not the only determinant of specificity of bond cleavage. The term 'chymotrypsin-like' activity therefore seems to be inappropriate.

The term 'peptidylglutamyl-peptide hydrolase' is also unsuitable on the basis of the inhibitor specificity shown here. Cbz-Tyr-Ala-Glu-CH₂Cl does not inhibit either the LLE1 or the LLE2 component, and Cbz-Leu-Leu-Glu-CH₂Cl only has a slight inhibitory effect. Both LLE1 and LLE2 components can be inhibited by some reagents containing hydrophobic residues in P1 and P2, and dipeptidyl derivatives seem to be adequate for binding to these sites. Studies of proteasome cleavage sites in peptide substrates (Wilk and Orlowski, 1980; Rivett, 1985; McDermott et al., 1991) have shown that cleavage can occur with many different amino acids in the P1 position, and it is not always easy to predict which catalytic sites are involved.

The trypsin-like activity of the rat liver proteasomes is easily distinguished from the others. Boc-Leu-Ser-Thr-Arg-NH-Mec is one of several suitable substrates the hydrolysis of which is inhibited by some peptidylchloromethane inhibitors containing an arginine residue in the P1 position. Tyr-Gly-Arg-CH₂Cl, in particular, has potential as a selective affinity label for trypsin-like activity (P. J. Savory, E. Shaw, K. S. Lilley and A. J. Rivett, unpublished work). The specificity for Arg in the P1 position is also suggested by the observed selective inhibition of this activity by peptidylarginine aldehyde inhibitors, leupeptin and antipain (Rivett, 1989b). However, it should be noted that peptide aldehyde inhibitors having hydrophobic residues in the P1 position can also inhibit this activity (Djaballah et al., 1992). Moreover, several peptidylchloromethanes with an arginine residue in the P1 position did not inhibit rat liver proteasomes or the rat muscle enzyme (Dahlmann et al., 1985), suggesting that there are additional structural requirements for binding to this site. The mechanism of the time-dependent stimulation of trypsin-like activity of rat liver proteasomes by some affinity-labelling reagents is not clear, but could be explained by modification of a different catalytic site or by an allosteric effect. A similar stimulation of trypsin-like activity of rat liver proteasomes has been noted previously with 3,4-dichloroisocoumarin (Djaballah et al., 1992). Also, a variety of reagents have been found to have

effects on activities of the bovine pituitary complex while binding at other sites (see, e.g., Cardozo et al., 1992).

Several of the inhibitors tested here will be useful in that they can selectively and irreversibly block some catalytic activities while having relatively little effect on others. The results confirm those of studies with serine-proteinase inhibitors, which suggest that rat liver proteasomes contain at least five distinct catalytic centres (Djaballah et al., 1992). The five activities which were assayed seem to be catalysed predominantly at distinct sites, although overlapping specificity of some catalytic centres of proteasomes would not be surprising. In the case of Suc-Leu-Leu-Val-Tyr-NH-Mec, the results suggest that the hydrolysis of this substrate occurs at more than one site, because some peptidylchloromethanes and peptidyl diazomethanes have a rapid, but limited, effect on activity. There may be additional catalytic centres which have not yet been identified.

The results of the present studies have provided further insight into the specificity of the different catalytic centres of proteasomes, and it is likely that some of the reagents will be useful in helping to unravel the functions of the many different subunits of the complex.

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