RESEARCH COMMUNICATION

Peptide glyoxals: a novel class of inhibitor for serine and cysteine proteinases

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A series of novel synthetic dipeptides, containing a C-terminal glyoxal grouping (–COCHO), have been tested as inhibitors against typical members of the serine- and cysteine-proteinase families. For example, the sequences benzyloxycarbonyl (Cbz)-Pro-Phe-CHO (I) and Cbz-Phe-Ala-CHO (II), which fulfil the known primary and secondary specificity requirements of chymotrypsin and cathepsin B respectively, have been found to be potent reversible inhibitors of their respective target proteinase. Thus I was found to inhibit chymotrypsin with a K_i of $\sim 0.8 \mu\text{M}$,

whereas II exhibits a K_i of $\sim 80 \text{ nm}$ against cathepsin B. These K_i values are some 10-fold and 3-fold lower than those reported for the corresponding peptide-aldehyde inhibitors of chymotrypsin and cathepsin B upon which the peptidyl-glyoxals were fashioned. Unexpectedly, the sequence Cbz-Pro-Ala-CHO, which was designed to inhibit elastase-like proteinases, exhibited no inhibitory activity towards porcine pancreatic elastase, even when used at concentrations as high as $200 \mu\text{M}$.

INTRODUCTION

Peptide sequences in which the C-terminal amide (–CONH–) or acid (–COOH) functional groups have been replaced by electrophilic moieties such as aldehyde (–CHO) [1,2], trifluoromethyl ketone (–COCF₃) [3,4] and α -oxo ester (–COCOOR) [5–7], have yielded potent reversible inhibitors of the serine and cysteine proteinases. By choosing appropriate amino acids to occupy the P₁ to P_n positions of the inhibitor (nomenclature of Schechter and Berger [8]), so as to fulfil the primary and subsite specificity requirements of individual members of these proteinase superfamilies, it has been possible to obtain reagents that exhibit pronounced selectivity of action.

As part of an ongoing programme aimed at the design of novel inhibitors for these two classes of proteinases (see, for example [9–11]), we have developed a range of novel dipeptides of general formula Cbz-NHCH(R²)CONHCH(R¹)COCHO (Cbz, benzyloxycarbonyl; R¹ and R² represent the side-chain groupings of naturally occurring amino acids), in which the C-terminal amino acid has been chemically transformed into an electrophilic α -amino glyoxal derivative –NHCH(R¹)COCHO. These putative inhibitors were synthesized from the corresponding peptidyl-diazomethanes [Cbz-NHCH(R²)CONHCH(R¹)COCHN₂], by oxidative cleavage of the diazo group, using dimethyldioxirane (DMD) [12].

The present paper reports on the kinetic analysis of the inhibition of typical members of the serine and cysteine proteinases by a series of these novel reagents.

EXPERIMENTAL

Materials

N-Benzyloxycarbonyl-L-arginyl-L-arginyl-4-methylcoumarin-7-ylamide (Cbz-Arg-Arg-NHMec) was purchased from Bachem, Bubendorf, Switzerland. Bovine cathepsin B, bovine chymo-

trypsin (thrice-recrystallized), porcine pancreatic elastase (thrice-recrystallized), *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-4-methylcoumarin-7-ylamide (Succ-Ala-Ala-Pro-Phe-NHMec) and *N*-methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valyl-4-methylcoumarin-7-ylamide (MeOSucc-Ala-Ala-Pro-Val-NHMec) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Human cathepsin B was extracted and purified from post-mortem liver, essentially as described by Barrett [13]. Samples of the purified proteinase, when subjected to SDS/PAGE run under reducing conditions, gave a major band of M_r 26000 and a faint low- M_r (~ 3000) band.

Synthesis of peptide glyoxals

The following peptide glyoxals were prepared as putative inhibitors: chymotrypsin-targeted sequences, Cbz-Ala-Phe-CHO, Cbz-Val-Phe-CHO and Cbz-Pro-Phe-CHO; cathepsin B-targeted sequence Cbz-Phe-Ala-CHO- and elastase-targeted sequence Cbz-Pro-Ala-CHO. The details for the synthesis of the dipeptide glyoxals will be published elsewhere [12]. In essence, they were prepared, in almost quantitative yield, by the DMD-catalysed oxidative cleavage of the parent peptidyl-diazomethane in moist acetone and were obtained as their hydrates [–COC(OH)₂], as confirmed by ¹H-n.m.r. and elemental analysis.

Kinetic techniques

Inhibition studies on chymotrypsin

Chymotrypsin ($10 \mu\text{l}$ of a $\sim 0.1 \mu\text{M}$ stock solution in 1 mM HCl) was added to a solution (1 ml) of Succ-Ala-Ala-Pro-Phe-NHMec ($50 \mu\text{M}$) and inhibitor under study (0.1 – $100 \mu\text{M}$) in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl , maintained at 37°C . The rate of hydrolysis of substrate was monitored continuously by measuring the rate of increase in

Abbreviations used: Cbz, benzyloxycarbonyl; Mec, 4-methylcoumarin-7-yl; MeO, methoxy; Succ-, succinyl (HO₂C–CH₂–CH₂–CO₂–); –Phe–H, –NH–CH–(CH₂–C₆H₅)–CHO; –Phe–CHO, –NH–CH–(CH₂–C₆H₅)–CO–CHO; –Ala–H, –NH–CH–(CH₃)–CHO; –Ala–CHO, –NH–CH–(CH₃)–CO–CHO; DMD, dimethyldioxirane.

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fluorescence at 455 nm (excitation wavelength 383 nm) in a Perkin-Elmer MPF 44B spectrofluorimeter.

Inhibition studies with pancreatic elastase

Pancreatic elastase (10 μ l of a ~ 0.1 μ M stock solution in 1 mM HCl) was assayed in the presence of MeOSucc-Ala-Ala-Pro-Val-NH-Mec (50 μ M) and inhibitor under study (10–200 μ M), exactly as described for chymotrypsin.

Inhibition studies with bovine and human cathepsin B

A solution (10 μ l) of cathepsin B (~ 50 nM) was added to a solution of (1 ml) of Cbz-Arg-Arg-NH-Mec (50 μ M) and Cbz-Phe-Ala-CHO (10–300 nM) in 100 mM sodium phosphate buffer, pH 6.4, containing 2 mM cysteine, 1 mM EDTA and 0.1% (w/v) Brij 35, maintained at 37 °C. The rate of substrate hydrolysis was monitored as described for chymotrypsin.

Determination of the operational molarity of the proteinase solutions

Chymotrypsin was titrated with the spectrofluorimetric titrant 4-methylumbelliferyl *p*-guanidinobenzoate by the method of Jameson et al. [14], whereas cathepsin B was titrated with accurate amounts of E-64 as described by Barrett and Kirschke [15]. The molarities of each of the proteinase solutions were then related to the steady-state hydrolysis of a solution (1 ml) of the respective fluorogenic substrates (both used at 20 μ M final concentration) for each proteinase, in order to give a more convenient measure of enzyme concentration from day to day.

Determination of K_m and V_{max} for the fluorogenic substrates

To determine the K_m and V_{max} for the substrates used in the present study, substrate concentrations spanning a range 0.2–5 times the K_m were used. For each enzyme/substrate pair studied, it was ensured that the determination of the kinetic constants were carried out under the exact conditions used to monitor the inhibition processes described above. K_m and V_{max} were determined by using the least-squares method of Roberts [16].

RESULTS AND DISCUSSION

Each of the glyoxals examined behaved as classical reversible inhibitors of their respective target proteinase. Table 1 lists the kinetic constants that were determined for the inhibition of

chymotrypsin and cathepsin B (human and bovine species) by these reagents. Also included for comparison, are the K_i values determined for the inhibition of chymotrypsin and cathepsin B by the aldehyde inhibitors Cbz-Val-Phe-H and Cbz-Phe-Ala-H respectively [3].

A number of points are noteworthy. First, in common with the peptide aldehydes, the glyoxal analogues are more potent inhibitors of the cysteine proteinases than they are of the serine proteinases. Thus the most potent inhibitor of chymotrypsin, Cbz-Pro-Phe-CHO, has a K_i of 0.8 μ M; this is to be compared with a K_i value of ~ 82 nM obtained for the inhibition of bovine cathepsin B by Cbz-Phe-Ala-CHO. This latter sequence was also a potent inhibitor of the human proteinase ($K_i \sim 77$ nM). Secondly, a comparison of the K_i values for the peptide aldehyde and their analogous glyoxal counterparts demonstrates that the latter are between 3- and 10-fold more potent inhibitors of their respective target proteinase than the former. We have endeavoured to carry out our inhibition studies under conditions as close as possible to those reported for the inhibition of the proteinases with the aldehyde inhibitors, and we are confident that the increase in potency obtained with our novel inhibitors is an accurate reflection of the relative potencies of the two classes of reagents.

Finally, the rank order of effectiveness observed within the chymotrypsin-specified sequences, Cbz-Pro-Phe-CHO > Cbz-Val-Phe-CHO > Cbz-Ala-Phe-CHO, is in keeping with the known subsite specificity requirements of the enzyme derived from previous substrate and inhibitor studies [3]. The inactivity of Cbz-Pro-Ala-CHO towards chymotrypsin (no inhibition observed using inhibitor concentrations up to 200 μ M) is also in keeping with the known primary specificity of the proteinase.

The only unexpected finding to arise out of the present study was the complete lack of inhibitory activity of Cbz-Pro-Ala-CHO towards porcine pancreatic elastase. This is difficult to explain in light of the reportedly strong inhibition of this proteinase by the tripeptide aldehyde derivative acetyl-Ala-Pro-Ala-H, for which a K_i of ~ 60 μ M was obtained [1]. However, it does suggest that the glyoxal derivatives may be very useful in discriminating between members of the serine-proteinase subclass.

As yet, we have not examined the exact mechanism for the inhibition of the serine or cysteine proteinases with these novel reagents. However, it seems reasonable to suggest that they will probably form hemiketals and thiohemiketal adducts with the active-site serine and cysteine residues of the respective classes of proteinase, in a manner similar to that established for the peptide aldehydes [17–20]. With the glyoxals, however, Ser¹⁹⁵ (chymotrypsin numbering) or Cys²⁵ (papain numbering) could conceivably add to either the ketonic or aldehydic carbonyl groups of the inhibitor. On the basis of electrophilic character, one would favour addition to the latter, but the former may be better placed, geometrically speaking, to interact with the active-site nucleophiles of these proteinases. The use of inhibitors incorporating ¹³C labels at each of these positions, used in conjunction with n.m.r., could presumably distinguish between these two mechanisms.

In conclusion, we have developed a novel class of inhibitors for the serine and cysteine proteinases, and early indications suggest that their potency surpasses even that of peptide aldehydes, which have previously been demonstrated to be potent inhibitors of these two classes of proteinase. On the basis of results from previous studies using peptide aldehydes [21], it seems reasonable to suggest that extension of the peptide glyoxal sequences so as to encompass the P₃ and P₄ subsites should result in the generation of inhibitors with improved potency.

Table 1 Inhibitor constants (K_i) for the interaction of peptide glyoxals and with chymotrypsin, elastase and cathepsin B

Proteinase	Inhibitor sequence	K_i^*
Chymotrypsin	Cbz-Ala-Phe-CHO	13.0 $\pm$ 1.5
	Cbz-Val-Phe-CHO	5.5 $\pm$ 0.6
	Cbz-Pro-Phe-CHO	0.85 $\pm$ 0.07
	Cbz-Val-Phe-H	54†
Cathepsin B		
	Cbz-Phe-Ala-CHO	76.8 $\pm$ 8.0
	Cbz-Phe-Ala-CHO	81.7 $\pm$ 8.5‡
	Cbz-Phe-Ala-H	210†
Porcine pancreatic elastase	Cbz-Pro-Ala-CHO	No inhibition

* For the chymotrypsin-directed sequences, K_i values are quoted in μ M; for cathepsin B-directed sequences, K_i values are quoted in nM.

† Data taken from Peet et al. [5].

‡ Values are means $\pm$ S.E.M. for four determinations.

Finally, we believe that further studies are warranted in order to examine more fully the mechanistic aspects of the inhibition and to extend the kinetic study to include the testing of peptide sequences targeted against the trypsin-like proteinases of the serine-proteinase subclass and the analogous clostripain-like cysteine proteinases.

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