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Active center differences between cathepsins L and B: the S_1 binding region

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The substrate peptide bond cleaved by cathepsins B and L is determined not by the amino acid contributing the carboxyl group to this bond as in the case of serine proteases but rather by the presence of a neighboring amino acid with a large hydrophobic side chain. From a study of the inhibitory potency in a series, Cbz-Phe-X-CHN_2, in which Phe promotes binding at S_2 (terminology of [(1968) Biochem. Biophys. Res. Commun. 32, 898–902]) while the amino acid X probes S_1, it is shown that this region of cathepsin L also has the ability to accommodate large hydrophobic side chains. In this respect cathepsin L differs from cathepsin B. Thus Cbz-Phe-Tyr(*O-t*-Bu)CHN_2 inactivates cathepsin L with a rate 2.5×10^4 greater than that for cathepsin B.

Cathepsin L; Cathepsin B; Selective inhibitor; Peptidyl diazomethyl ketone

1. INTRODUCTION

The protease, cathepsin L, initially isolated from rat liver lysosomes [2] is typically a 2-chain protease in its mature form [3]. It appears to be the most active of the lysosomal cysteine proteases, which include cathepsin B, H and possibly others [4]. The role of cathepsin L is probably not limited to lysosomal protein degradation since precursor forms are secreted by fibroblasts upon transformation [5,6] and by stimulated inflammatory macrophages [7]. Since cathepsin B has also been found as incompletely processed forms [8,9], interest in improved methods for the biochemical study of these proteases has increased. Cathepsin L cleaves proteins near hydrophobic amino acid residues which bind to subsites S_2 and S_3 [10,11], a specificity similar to cathepsin B [4] and attempts to devise selective substrates based on these properties have not yet met with success [11].

Synthetic inhibitors have been more useful in discriminating between cathepsin B and L in vitro and also in blocking their action in vivo. Thus, Cbz-Phe-AlaCHN_2 readily inactivates both proteases [12–14] but Cbz-Phe-PheCHN_2 is more effective against cathepsin L [14]. It is evident that a phenylalanine residue in P_2 promotes binding to both proteases, but that a difference exists in S_1 with respect to the size of side chain that can be accommodated. This region of cathepsin B was explored earlier in a series of the general structure, Cbz-Phe-XCHN_2, in which the amino acid, X, had side chains of differing bulk [15] to provide probes. Differences in reactivity up to 10^4 were found due to steric limitations in the active center of cathepsin B. These inhibitors have now been examined with cathepsin L in the hope of obtaining similar information and perhaps finding useful differences in reactivity of the two proteases.

2. MATERIALS AND METHODS

Cathepsin L was isolated from rat liver [2] and stored as the mercury salt [4]. The stock enzyme was 54 μM by titration; 1:100 dilutions, activated with thiol-containing buffer [4] at 25°C for about 10 min, were used to prepare reaction mixtures

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with [E] = 1.1 nM and inhibitor at 25°C. Timed aliquots were removed for the assay in a recording spectrofluorimeter at 37°C using Cbz-Phe-Arg aminomethylcoumarinylamide [4], 4×10^{-5} M, in the same buffer.

The peptidyl diazomethyl ketones have been described [15] except for Cbz-Phe-Tyr(*O*-*t*-Bu)CHN_2, m.p. 137–138°C, obtained in the usual way [15]. Prior to use, millimolar stock solutions in acetonitrile were prepared and diluted with buffer to provide working solutions such that the final organic solvent was 1% (v/v) or less. Dilutions of inhibitor were favored that gave relatively slow inactivations to avoid saturation effects and to permit the assay of 4–10 aliquots. With extremely effective inhibitors, i.e., causing inactivations in the nanomolar range, this was barely possible since [I] was approaching [E]. However, in the studies reported here, the loss of enzymatic activity followed 1st-order kinetics as seen previously, for example, in the inactivation of cathepsin B by Cbz-Phe-PheCHN_2 [12].

3. RESULTS AND DISCUSSION

The inhibitors of this group were in general more effective in inactivating cathepsin L than B. The rates of the more active ones (table 1) are about 100-fold greater for cathepsin L than for B [15], although the range of values is narrower for L. This reflects restricted binding of large substituents in the S_1 region of the active center of cathepsin B [15] whereas L can accommodate such substituents readily. It is evident that some of the poorer inactivators for cathepsin B are very effective against cathepsin L and thus may be useful for discriminating between these proteases. A selection of the more specific inhibitors of cathepsin L is given in table 2 for a comparison of the rates of inactivation. Differences of 10^4 are evident as in the case of Cbz-Phe-Tyr(4-*O*-*t*-Bu)CHN_2. This is considerably better than the selectivity provided by Cbz-Phe-PheCHN_2 [4].

Table 1

The effect of amino acid, X, in the reagent Cbz-Phe-X-CHN_2, on the inactivation of cathepsin L, pH 5.4, 25°C

X	[I] (M)	$t_{1/2}$ (min)	k_{app} (min^{-1})	Inactivation rate ($M^{-1} \cdot min^{-1}$)
Citrulline	1.5×10^{-9}	4.1	0.169	0.113×10^9
Ser(-OBzl)	5×10^{-9}	2.3	0.301	0.060×10^9
Cys(S-Bzl)	5×10^{-9}	5.9	0.117	0.0234×10^9
Phe(4-NO_2)	5×10^{-9}	11.8	0.0587	0.0117×10^9
Tyr(*O*-*t*-Bu)	1×10^{-8}	5.7	0.122	0.122×10^8
Tyr(*O*-Me)	1×10^{-8}	6.5	0.107	0.107×10^8
Trp	2×10^{-8}	4.1	0.169	0.845×10^7
2-Naph-Ala	2×10^{-8}	5.3	0.131	0.655×10^7
Thr(*O*-*t*-Bu)	2×10^{-8}	9.5	0.073	0.365×10^7
homo-Phe	2×10^{-6}	10.8	0.0642	0.0321×10^6

Table 2

Reagents which are much more effective against cathepsin L than B

Residue X in Cbz-Phe-X-CHN_2	Rate of inactivation of		Relative rate
	Cathepsin L ($M^{-1} \cdot min^{-1}$)	Cathepsin B ($M^{-1} \cdot min^{-1}$)	
Tyr(*O*-*t*-Bu)	1.2×10^7	0.62×10^3	2.5×10^4
Tyr(*O*-Me)	1.1×10^7	1.55×10^3	0.6×10^4
Phe(4-NO_2)	1.2×10^7	3×10^3	0.4×10^4
Trp	0.85×10^7	1.2×10^4	0.7×10^3

The advantages of peptidyl diazomethyl ketones for the inactivation of the lysosomal cysteine proteases include the ability of these reagents to penetrate cells of diverse types and inactivate the proteases [16–18]. Leupeptin has sometimes been used for this purpose but lacks selectivity since it inhibits both serine and cysteine proteases [19], in contrast to the peptidyl diazomethyl ketones, including not only the lysosomal cathepsins [4] but also the calcium-activated proteases [20]. Possibly because of this, leupeptin administered to animals actually increases cathepsin L in various organs [20–22] probably by inhibiting protease degradation. This has been ascribed to a role of the lysosomal cysteine proteases in their turnover [24] due to a similar effect of Ep-475, an epoxide inactivator of cysteine proteases [25]. On the other hand, a more specific inhibitor, Cbz-Phe-AlaCHN_2 does not produce this elevated proteolytic activity in animals [23].

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