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RAPID INACTIVATION OF CATHEPSIN L BY Z-PHE-PHECHN$_2$[1] AND Z-PHE-ALACHN$_2$

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SUMMARY

Z-Phe-PheCHN$_2$ and Z-Phe-AlaCHN$_2$ were found to react extremely rapidly with cathepsin L[2] from rat liver lysosomes. For measuring the inactivation time it was necessary to work with dilute enzyme and inhibitor solutions, that is in the range of 10^{-9} M. These conditions were made possible through the use of Z-Phe-Arg-4-methyl-7-coumarylamide, a very sensitive substrate suitable for assays of cathepsin L in about 10^{-12} M solutions.

Z-Phe-AlaCHN$_2$ has an affinity for cathepsin L which is about 2000-fold higher than for cathepsin B from rat and human.

Z-Phe-PheCHN$_2$ proved to be a selective inactivator of cathepsin L in a certain concentration range. Z-Phe-PheCHN$_2$ reacts reversibly with cathepsin B from several species.

It has been found that peptidyl diazomethyl ketones are selective inhibitors of cysteine proteinases (1-4). They do not inactivate serine or metallo proteinases and their inhibitory effect on aspartate proteinases requires copper ion (5,6).

The rate of reaction of a cysteine proteinase with various peptidyl diazomethyl ketones depends on the specificity of the enzyme (1,4) therefore there appears to be some possibility of obtaining selective inhibitors for individual cysteine proteinases. Three cysteine proteinases, cathepsins B, L, and H occur in lysosomes. To explore the possible physiological role of each of them selective inhibitors are necessary.

Cathepsin H is an endoaminopeptidase (7,8) and its specificity has not been studied in detail. Cathepsin B and cathepsin L have some similarities in their specificity (hydrolysis of Z-Lys nitrophenyl ester, benzoyl-Arg amide, and Z-Phe-ArgNMec, collagenolytic activity, inactivation of aldolase, glucokinase and pyruvate kinase, some common split positions in the insulin B chain). On the other hand, there also some differences in their substrate

[1] Abbreviations: Z- = benzyloxycarbonyl, NMec = 4-methyl-7-coumarylamide, NNap = 2-naphthylamide, HO-Eps- = trans-epoxysuccinoyl-. The usage of P_1 etc. is based on the convention introduced by Schechter, I. and Berger, A. Biochem. Biophys. Res. Communs. (1967), 27, 157-162.

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specificity. Bz-ArgNNap and Z-Arg-ArgNNap, very sensitive substrates for cathepsin B (9), are only slowly hydrolyzed by cathepsin L (10). Aldolase and glucagon are split by cathepsin B in a peptidyl dipeptidase mode of action (11, 12) whereas cathepsin L acts on these substrates as an endopeptidase (13). Most preferential split positions in a polypeptide chain for the action of cathepsin L are those with hydrophobic amino acids in P_1, P_2 and P_3 and those with hydrophobic amino acids in P_2 and P_3 only (14).

We had observed earlier that Z-Phe-PheCHN$_2$ and Z-Phe-AlaCHN$_2$ are very effective in inactivating cathepsin L (10) but it was not possible to measure the rate of inactivation because of the lack of an assay method capable of use with extremely dilute cathepsin L solutions. Very recently Z-Phe-Arg 4-methyl-7-coumarylamide (Z-Phe-ArgNMec) has been found to be a very sensitive substrate not only for cathepsin B, but also for cathepsin L, suitable for assays of cathepsin L in about 10^{-12} M solutions (15). Quantitative evaluation of these inhibitors has now been carried out.

METHODS

Reagents: The synthesis of the inhibitors used in this investigation has been described (2). Z-Phe-ArgNMec, 7-amino-4-methyl-coumarin, HO-Eps-Leu-amido(3-methyl)butane (E-475) and human cathepsin B (16) were kindly given by Dr. A.J. Barrett; Z-Arg-ArgNNap, by Dr. C.G. Knight. Dimethylsulfoxide was purified and kindly provided by Doz. Dr. S. Fittkau. Dithioerythritol was obtained from Serva, Heidelberg.

Enzymes: Cathepsins B,H and L from rat liver were prepared as described previously (7, 17). The molar concentration of the active sites of cathepsins B and L was determined by titration with E-475 (18). This reagent is an analog of the thiol protease inhibitor from mold described by Hanada et al. (19).

Inactivation studies: Cathepsin L was diluted with 20mM phosphate buffer pH 6.0, 5 mM in dithioerythritol and 2.5 mM in EDTA to give a 2.8×10^{-9} M solution. Cathepsin B (rat) was 2.2×10^{-8} and cathepsin B (human) was 2.5×10^{-8} M in 20 mM phosphate buffer pH 6.0, 5 mM in dithioerythritol and 2.5 mM in EDTA. Cathepsin H was used as a 3×10^{-6} M solution. Stock solutions of the inhibitors 0.1 M in pure dimethylsulfoxide were diluted stepwise with 20 mM phosphate buffer pH 6.0 containing 0.1% Triton X-100. 100 µl of the enzyme solutions (of the given concentration) were added at room temperature to 100µl of the inhibitors at the appropriate concentration. 10µl of the reaction and control mixture without inhibitor were assayed at various times for determining the extent of enzyme inactivation.

Assay: Cathepsins B and L were assayed with Z-Phe-ArgNMec as described by Barrett (20). 300ul of 20 mM phosphate buffer pH 6.0, 2 mM in dithioerythritol and 1 mM in EDTA were mixed at 40° C with 200 µl 12.5 uM Z-Phe-ArgNMec in 20 mM phosphate buffer (freshly diluted from a 10 mM stock solution in dimethyl sulfoxide). The reaction was started with 10 µl of the inactivation mixture and stopped after 10 minutes at 40°C by addition of 500 µl of 100 mM chloroacetate in acetate buffer pH 4.3. Hydrolysis of the substrate was determined fluorimetrically (1 ml cuvettes) using an Eppendorf fluorimeter with excitation at 366 nm and emmission measured at 430-470 nm. In some experiments Z-Lys nitrophenyl ester was used as a substrate for cathepsins B and H (1) and Z-Arg-ArgNNap for cathepsin B (9).

Table I

Rates of Inactivation of Catheptic Thiol Proteases by Peptidyl Diazomethyl Ketones

Enzyme[a]	Inhibitor[a]	Concentration (M)	$t_{1/2}$ (sec)	$k_{app}/I\ M^{-1}s^{-1}$
Cathepsin L (1.4×10^{-9} M)	Z-Phe-PheCHN$_2$	5×10^{-9}	210	660,000
	Z-Phe-AlaCHN$_2$	5×10^{-9}	224	620,000
Cathepsin B Rat liver (1.1×10^{-8} M)	Z-Phe-PheCHN$_2$	Reacts reversibly[b]		
	Z-Phe-AlaCHN$_2$	5×10^{-6}	276	500
Cathepsin B Human liver (1.25×10^{-8} M)	Z-Phe-PheCHN$_2$	Reacts reversibly[b]		
	Z-Phe-AlaCHN$_2$	5×10^{-6}	220	630
Cathepsin B[c] Beef spleen	Z-Phe-PheCHN$_2$	5×10^{-6}	1422	193
Cathepsin H (1.5×10^{-6} M)	Z-Phe-AlaCHN$_2$	5×10^{-4}	2190	0.6

[a] The concentration of enzyme and inhibitor refers to the reaction mixture. Cathepsins L and H are from rat liver.

[b] Z-Phe-PheCHN$_2$ was used in concentrations of 5×10^{-4} to 5×10^{-5} M. In this range incubation mixtures contained 0.5% dimethyl sulfoxide and 0.1% Triton X-100. In these cases the kinetics of reaction are more complex since there is a definite time-dependent inactivation which involves a reversible phase as well as some eventual irreversible binding (Kirschke and Fittkau, to be published).

[c] From ref. 1.

RESULTS AND DISCUSSION

Z-Phe-PheCHN$_2$ and Z-Phe-AlaCHN$_2$ react very rapidly with cathepsin L (Table 1). The time of inactivation was measurable only in very dilute solutions of enzyme and inhibitor. The half times for inactivation were read from semilogarithmic plots of residual activity versus time. Z-Phe-PheCHN$_2$ and Z-Phe-AlaCHN$_2$ proved to be the most effective inhibitors of cathepsin L described until now. Some other diazomethyl ketones have also been tested, but their reaction with cathepsin L was slow. The following peptidyl derivatives inhibit cathepsin L at a diminishing rate: Z-Lys-, Z-Ala-Ala-, Z-Gly-Gly-Leu-, Z-Gly-Gly-Ala-, Z-Gly-Gly-Phe-, Z-Gly-Gly-Val-, Z-Pro-Gly-, and Z-Gly-Gly-Pro diazomethyl ketones. Binding of compounds with hydrophobic residues in P_2 and P_3 (the benzyloxycarbonyl group is regarded as a hydrophobic residue) is very rapid, in accordance with the substrate specificity revealed by the sites of proteolysis in the insulin B-chain (14) and to a certain extent in glucagon (13).

Z-Phe-AlaCHN$_2$, which seems to enter the cell by pinocytosis, has been found to suppress proteolysis in cultured macrophages (6). In view of the foregoing results this effect must be due not only to inhibition of cathepsin

B but also to cathepsin L as had been suggested (6). Z-Phe-AlaCHN$_2$ reacts with cathepsin L about 2000-fold faster than with cathepsin B (Table 1). On the other hand, this inhibitor is about 1000-fold more effective on cathepsin B than on cathepsin H.

The order of effectiveness of some peptidyl diazomethyl ketones in inactivating cathepsin B (in decreasing rate) is in contrast to the order of their effectiveness on cathepsin L: Z-Phe-Ala-, Z-Phe-Gly-, Z-Ala-Ala-, Z-Lys-, Z-Phe-Phe-.

Z-Phe-PheCHN$_2$ proved to be a reversible inhibitor of cathepsin B from the rat. The extent of inhibition was independent of the incubation time (5 min to 3 hr) and was dependent only on the concentration of Z-Phe-PheCHN$_2$: 5×10^{-4}M caused 62.5%, 2.5×10^{-4}M, 55.5% and 5×10^{-5}M , 19.7% inhibition of cathepsin B. We did not find competition with the substrate (which in these experiments was Z-Arg-ArgNNap). Preliminary observations with human cathepsin B also indicate that Z-Phe-PheCHN$_2$ is acting as a reversible inhibitor. In contrast, this reagent is an irreversible inhibitor of beef spleen cathepsin B (1,3). Further experiments will clarify to what extent species differences lead to different modes of reaction. Nevertheless, low concentrations of Z-Phe-PheCHN$_2$ allow the selective inactivation of cathepsin L in a mixture with cathepsins B and H. The low solubility and possible mutagenic properties may limit the application of this inhibitor in vivo.

We can conclude from our results that peptide derivatives with three hydrophobic residues have a high affinity for cathepsin L On the other hand, a large hydrophobic side chain in P_1 probably prevents effective binding to the active center of cathepsin B.

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