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The affinity-labelling of cathepsin S with peptidyl diazomethyl ketones

Comparison with the inhibition of cathepsin L and calpain

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Since peptidyl diazomethyl ketones are useful irreversible inhibitors for inactivating cysteinyl proteinases in vitro and in vivo and in order to reveal their role, we set out to obtain selective and effective reagents for cathepsin S. A number of such derivatives with hydrophobic amino acid residues, such as valine, leucine and tryptophane in positions adjacent to the primary specificity site were synthesized and these provided inhibitors rapidly acting at high dilution. For example, 1 nM Z-Leu-Leu-Nle-CHN_2 inactivates cathepsin S with $k_{2nd} = 4.6 \times 10^6\ M^{-1} \cdot s^{-1}$ at pH 6.5, 25°C. Similarities to the specificities of cathepsin L and calpain were evident. However, Z-Val-Val-NleCHN_2 is over 300 times more effective in inactivating S than L. On the other hand, Z-Phe-Tyr(t-Bu)CHN_2 is about 10^4 more effective against L than S. Reagents are thus now available for a clear discrimination between these proteases.

1. INTRODUCTION

Cathepsin S is a cysteinyl proteinase originally purified from bovine lymph nodes [1] and spleen [2–6], and was later found in kidney [7]. Confirmation of the distinction of this protease from cathepsin L [3] was initially provided by differences in proteolytic specificity [4], immunochemical properties and susceptibility to inhibitors [3,8]. Studies on the collagenolytic proteases of rabbit spleen have also led to a similar conclusion [9,10]. Eventually the determination of the sequences of the bovine [11,12] and human [13,14] proteases established the distinct identity of cathepsin S.

In the course of this work, the irreversible inhibitor, Z-Phe-Phe-CHN_2 was useful since its known inactivation of cathepsin L at μM concentrations [8,15] was not matched in the case of cathepsin S [3,7], but a specific inhibitor for cathepsin S has not been available. The peptidyl diazomethyl ketones are inactivators of cysteinyl proteases [16] and sequence variations can generally provide some degree of specificity due to differences in their substrate-binding regions. They apparently owe their effectiveness to a substrate-like interaction of the peptide ketone group with the active-center -SH group, to provide an initial enzyme–inhibitor complex, a transition-state analog [17]. We felt that a systematic study with cathepsin S would probably yield more effective inhibitors and provide information about the substrate binding region.

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Abbreviations: AMC, 4-methyl-7-coumarylamide; t-Leu, $(CH_3)_3C$-$CH(NH_2)$-COOH; γ-Me-Leu, $(CH_3)_3C$-CH_2-$CH(NH_2)$-COOH; Z-, Carbobenzyloxy-.

2. MATERIALS AND METHODS

The peptidyl diazomethyl ketones were obtained from the corresponding peptide by reaction of its mixed anhydride with ethereal diazomethane as described [16,18]. The products were purified by chromatography on silica gel with chloroform containing increasing amounts of methanol. The purified inhibitors were generally obtained as solids, recrystallized, and their compositions confirmed by elementary analysis.

The new materials are Z-Leu-AlaCHN_2, M.P. 90–91; Z-Leu-Leu-NleCHN_2, M.P. 143–144; Z-Ile-MetCHN_2, M.P. 143–144; Z-Trp-MetCHN_2, M.P. 153–154; Z-Val-MetCHN_2, M.P. 144–145; Z-Val-ValCHN_2, M.P. 134–136; Z-Val-NleCHN_2 M.P. 140–141; Z-Val-Val-NleCHN_2, M.P. 244–247; Z-Val-Trp-NleCHN_2, M.P. 177–178; Z-Val-Val-TyrCHN_2, M.P. 170–172; and Z-Ile-TrpCHN_2, M.P. 156–157. Z-Leu-NvaCHN_2, Z-Leu-NleCHN_2, and Z-Leu-Thr(tBu)CHN_2 did not crystallize and their structures were confirmed by mass determination. Z-Phe-PheCHN_2 [18], Z-Phe-Tyr(tBu)CHN_2 [20], Z-Leu-LeuCHN_2 [19], Z-Leu-TrpCHN_2 [19], Z-Leu-Leu- TyrCHN_2 [19] and Z-γ-methylLeu-TyrCHN_2 [19] were obtained as described. Z-Leu-Nle-aldehyde was purchased from Nova.

For the evaluation of inhibitory properties, inhibitors were made up as 10^{-2} M solutions in DMSO which usually could be diluted with water or 5% DMSO without precipitation. In the enzymatic studies the DMSO content was typically well below 1%.

Cathepsin S was purified from bovine spleen [21] and activated before use with 5 mM DTT in 0.1 M PIPES buffer, pH 6.5, 75 mM in NaCl, 1 mM in EDTA, 0.01% Triton X-100 for 15 min at room temperature, then diluted 1:3 with buffer without DTT and stored on ice. It was assayed fluorimetrically with 4×10^{-5} M Z-Phe-Arg-AMC at 37°.

Cathepsin L was purified from bovine kidney [21] and was activated

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before use [22]. It was assayed with 2×10^{-5} M Z-Phe-ArgAMC in 0.1 M acetate, pH 5.4, 1 mM EDTA, 0.01% Brij 35 and 1 mM DDT.

Inhibitions were followed by removing timed aliquots from a mixture of inhibitor and cathepsin S at room temperature and the decay of enzyme activity was measured under first order conditions [18] in a range of inhibitor concentrations that avoided saturation effects. Replicate determinations were carried out and the results were within a 15% variation from the mean. In the case of Z-Val-ValCHN$_2$ a Dixon plot [23] was used to calculate K_i by measuring initial velocities in mixtures of the inhibitor and 2×10^{-5} and 8×10^{-5} M Z-Phe-ArgAMC at 37°. The K_i of Z-Leu-Nle-Aldehyde was similarly determined, but the higher substrate concentration was 10^{-4} M.

3. RESULTS AND DISCUSSION

Our earlier work on the inactivation of cysteinyl proteinases with peptidyl diazomethyl ketones was directed largely at cathepsins B and L [15,20] and the calcium-activated proteinase, calpain [19]. In terms of their specificities, the first two, cathepsin B and L, resemble the classical cysteinyl proteinase, papain, in that they effectively bind an aromatic residue in the S_2 site of the substrate binding region, making use of the terminology of Schecter and Berger [24,25] who first demonstrated this binding preference. When the specificity of calpain was explored with inhibitors, it was found that the S_2 binding region does not show affinity for aromatic side chains, but has a preference for aliphatic side chains such as leucine. Cathepsin L, in contrast, is susceptible to inhibitors which contain either an aromatic residue or leucine in the P_2 position [19].

Our observations with cathepsin S and peptidyl diazomethyl ketones began with Z-Phe-PheCHN$_2$ whose relatively low reactivity, 900 $M^{-1} \cdot s^{-1}$ for the enzyme from bovine spleen [6] was confirmed. The possibility was considered that this reflected a poor ability to bind an aromatic side chain in the S_1 and/or S_2 subsites as has been observed with Phe [15] or Trp [20] in P_1 in the case of cathepsin B or with aromatic residues in P_2 in the case of calpain [19]. When a single aromatic residue was present in P_1 (even Trp) a modest reactivity was observable as in the case of Z-Leu-TrpCHN$_2$ (Table I) and, in P_2, a favorable binding was evident, cf. Z-Trp-MetCHN$_2$ compared to Z-Val-MetCHN$_2$ (Table I). Thus, no difficulty in binding at least an inhibitor with a single aromatic amino acid was evident.

Table I

Rates of Inactivation of Cathepsin S, pH 6.5

Diazomethyl Ketone	Conc (M)	$t_{1/2}$ (min)	k_{app} (s^{-1})	k_{2nd} ($M^{-1} \cdot s^{-1}$)
Z-Leu-Leu-NleCHN$_2$	1×10^{-9}	1.25	0.0092	9.2×10^6
Z-Val-Val-NleCHN$_2$	1×10^{-9}	2.5	0.0046	4.6×10^6
Z-Leu-Leu-TyrCHN$_2$	1×10^{-9}	5.5	0.0021	2.1×10^6
Z-Trp-MetCHN$_2$	1×10^{-8}	3.0	0.00385	3.8×10^5
Z-Val-Val-TyrCHN$_2$	5×10^{-9}	6.75	0.0017	3.4×10^5
Z-Leu-NleCHN$_2$	1×10^{-8}	3.4	0.0034	3.4×10^5
Z-Leu-NvaCHN$_2$	1×10^{-8}	4.5	0.0026	2.6×10^5
Z-Val-Trp-NleCHN$_2$	1×10^{-8}	5.7	0.002	2.0×10^5
Z-Leu-LeuCHN$_2$	2×10^{-8}	8.6	0.0013	6.7×10^4
Z-Val-NleCHN$_2$	3×10^{-8}	5.8	0.0020	6.6×10^4
Boc-Lys-Leu-TyrCHN$_2$	1×10^{-7}	2.9	0.004	4.0×10^4
Z-Val-MetCHN$_2$	1×10^{-7}	3.5	0.0033	3.3×10^4
Z-Leu-TrpCHN$_2$	2×10^{-7}	2.6	0.0044	2.2×10^4
Z-Leu-AlaCHN$_2$	1×10^{-7}	5.5	0.0021	2.1×10^4
Z-γ-MeLeu-TyrCHN$_2$	4×10^{-7}	3.85	0.003	7.5×10^3
Z-Leu-Thr(t-Bu)CHN$_2$	1×10^{-6}	3.5	0.0033	3.3×10^3
Z-Val-ValCHN$_2$	1×10^{-7}	47.0	0.000246	2.5×10^3
Z-Ile-MetCHN$_2$	1×10^{-6}	5.4	0.0021	2.1×10^3
Z-Phe-PheCHN$_2$	2×10^{-6}	6.4	0.0018	9.1×10^2
Z-Ile-TrpCHN$_2$	4×10^{-6}	3.45	0.0033	8.4×10^2
Z-t-Leu-TyrCHN$_2$	1×10^{-4}	1.6	0.0072	72
Z-Phe-Tyr(t-Bu)CHN$_2$	2×10^{-5}	19.25	0.0006	30

We then examined inhibitors containing aliphatic side-chains, both straight-chained and branched, in P_1, P_2, and P_3. In P_1 amino acids with linear side chains such as norvaline, methionine, norleucine provide inhibitors with good reactivity as shown by a number of entries in Table I.

The P_2 variations provided some favorable results (Table I) with Leu, Val, and Trp, but less with Ile, γ-methyl-Leu, and t-Leu. For example, Z-Trp-MetCHN$_2$ inactivates cathepsin S with a $k_{2nd} = 3.8 \times 10^5$ $M^{-1} \cdot s^{-1}$ in contrast to Z-Ile-MetCHN$_2$, for which $k_{2nd} = 2.1 \times 10^3$ $M^{-1} \cdot s^{-1}$. The effectiveness of leucine in P_2 is reminiscent of results with cathepsin L [19]; the affinity of valine for cathepsin S was indicated in the work of Brömme et al. [8] with a number of substrates and inhibitors. Val and Leu seem almost equally effective in P_2 and P_3 as evidenced by our results with tripeptide derivatives (Table I), several of which act rapidly at nM concentrations.

The low reactivity of Z-Val-Val-CHN$_2$ was unexpected. This is not due to poor affinity to cathepsin S since competition of substrate hydrolysis [23] showed that this derivative is a good reversible inhibitor with $K_i = 0.9$ μM. This value is in the middle of the range of K_i values (0.3–2.2 μM) measured for six inhibitors of cathepsin L [19]. It may be that this substance is bound largely at S_2 and S_3 and thus the diazomethylketone group is too remote to interact with the -SH group.

Our studies have examined a limited number of sequences but the results demonstrate the large variation of inhibitory effectiveness for cathepsin S of structures constructed with peptidyl diazomethyl ketones. This study converges with earlier work in which cathepsin L and calpain [19] were shown to be susceptible to derivatives containing Leu in P_2 and P_3. However, there remain some useful distinctions. Cathepsin S was found to be resistant to Z-Phe-Tyr(t-Bu)CHN$_2$ and this substance remains a unique inhibitor of cathepsin L. Cathepsin S will bind an aromatic residue in S_2 like cathepsin L and in dinstinction to calpain.

Some similarities and differences in the response of cathepsins L and S to peptidyl diazomethyl ketones are listed in Table II. Although not all of the new derivatives have been tested with cathepsin L, it is of interest

Table II

Some of the major differences and similarities in the rates of inactivation of cathepsin S and L by peptidyl diazomethyl ketones

	Inactivation rate ($M^{-1} \cdot s^{-1}$)	
Similarities	Cathepsin S (pH 6.5)	Cathepsin L (pH 5.4)
Z-Leu-Leu-TyrCHN$_2$	2.1×10^6 (a) 1.3×10^5 (c)	1.5×10^6 (b)
Z-Trp-MetCHN$_2$	3.8×10^5 (a)	1.4×10^5 (a)
Z-Leu-TrpCHN$_2$	2.2×10^4 (a)	1.2×10^4 (b)
Differences		
Z-Tyr-AlaCHN$_2$	1.7×10^3 (d)	1.2×10^5 (d)
Z-Val-Val-NleCHN$_2$	4.6×10^6 (a)	1.2×10^4 (a)
Z-Phe-Tyr(t-Bu)CHN$_2$	30 (a)	2×10^5 (e)

(a) This work; (b) ref. 19; (c) ref. 27; (d) ref. 28; (e) ref. 20.

that Z-Val-Val-NleCHN$_2$ is considerably more effective (380-fold) in inactivating cathepsin S than L.

Radiolabelling of active cysteinyl proteinases in cells and tissues using ^{125}I derivatives of Tyr-containing diazomethyl ketones has become a useful tool in their study. The use of Z-[^{125}I]Tyr-AlaCHN$_2$ to label cathepsins B, S, and L [28,29] and Z-Leu-Leu-[^{125}I]TyrCHN$_2$ to label calpain and cathepsin L, and S [30,31] can be made more selective by the use of control inhibitors. Our results indicate that if the presence of both cathepsins S and L are a problem, prior treatment with Z-Phe-Tyr(t-Bu)CHN$_2$ will eliminate L and will leave functioning cathepsin S available for observation and iodination. It has to be kept in mind when using irreversible inhibitors on cells, that kinetic differences may disappear with high concentrations and long incubations. Further discussion of the combined use of inhibitors may be found in [26].

An independent line of study involving peptidyl aldehydes as reversible inhibitors of calpain has led to Z-Leu-Nle-aldehyde [32] and similar structures with hydrophobic side chains [33]. In view of this type of peptide portion, it seemed probable to us that these would also inhibit cathepsin S, as has been shown earlier for cathepsin L [34]. In fact, we found Z-Leu-Nle-aldehyde to be an effective inhibitor of cathepsin S, with $K_i = 2 \times 10^{-8}$ M (ave. of 4 runs). Therefore the results obtained with such inhibitors and the interpretation of these results to demonstrate a role for calpain should be reexamined. Such reagents could be used in conjunction with irreversible inhibitors as discussed above to obtain a more secure interpretation of their site of action.

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