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INHIBITION OF AMINOPEPTIDASE M BY ALKYL D-CYSTEINATES

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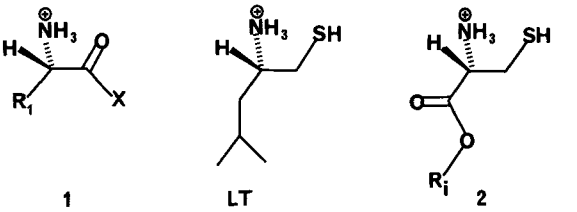
Ethyl D-cysteinate is a potent competitive inhibitor ($K_i = 3.5 \times 10^{-7}$ M) of aminopeptidase M. D-cysteine and ethyl L-cysteinate inhibit more than two orders of magnitude less effectively. Inhibition studies on several n-alkyl esters of D-cysteine reveal an optimum at the n-butyl ester ($K_i = 1.8 \times 10^{-7}$ M). The results are consistent with the hypothesis that the thiol group coordinates to Zn^{+2} at the active site and the alkyl group occupies the hydrophobic binding site for the side chain of the amino-terminal residue of substrates. Cytosolic leucine aminopeptidase is not significantly inhibited by ethyl D-cysteinate.

KEY WORDS: Aminopeptidase M, alkyl D-cysteinate, metallopeptidase.

INTRODUCTION

Aminopeptidase M from porcine kidney microsomes (EC 3.4.11.2) is a Zn^{+2}-containing enzyme that catalyzes the hydrolysis of the C–X bond in compounds of structure **1**. Substrates include both peptides and simple amides, and the Zn^{+2} is required for



1 **LT** **2**

catalytic activity.[1,2] Relatively little is known about the active site and catalytic mechanism of aminopeptidase M, but interest in this enzyme has been stimulated by recent evidence that a cerebral enzyme that is similar or identical to aminopeptidase M plays a major role in the degradation of enkaphalins.[3–5]

Chan and coworkers have reported[6,7] that L-leucinethiol (LT) is a powerful competitive inhibitor ($K_i = 5.1 \times 10^{-8}$ M) of aminopeptidase M. The most likely reason for the strong inhibition is that the sulfur atom in LT coordinates to the Zn^{+2} while the amino group and alkyl chain of the inhibitor occupy the binding sites for the amino group and R_1 of substrates. This explanation is consistent with the chemically attractive hypothesis that the function of the Zn^{+2} is to co-ordinate to the oxygen atom of the scissile carbonyl group of the substrate in order to make the carbon more

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Abbreviations: LT, leucinethiol; Tris, tris-(hydroxymethyl)aminomethane; LAP, leucine aminopeptidase.

susceptible to nucleophilic attack.[8] The fact that LT inhibits about two orders of magnitude more effectively than 2-aminoethanethiol[7] implies that the binding site for R_1 is hydrophobic. Relative K_m values for simple amide substrates also support this notion.[9]

In this paper we report that several alkyl esters of D-cysteine (**2**) are very good inhibitors of aminopeptidase M. Our rationale for investigating these compounds was that if the amino and thiol groups of **2** are bound as proposed for LT, the D-configuration at the α-carbon of **2** would enable R_1 to occupy the lower portion of the binding site for R_1. Variation of R_1 would then permit systematic optimization of the inhibition with a minimum of synthetic effort. More generally, by appropriate choice of R_1 it might be possible to develop inhibitors that would discriminate among different metal-dependent aminopeptidases with different specificities toward the amino-terminal residue.

MATERIALS AND METHODS

Aminopeptidase M, cytosolic leucine aminopeptidase, D-cysteine and ethyl L-cysteinate were obtained from Sigma Chemical Company. Ethyl D-cysteinate hydrochloride, mp = 125–127° (lit. mp of L-enantiomer = 128°)[10] was prepared by the acid-catalyzed esterification of D-cysteine by the procedure of Borders *et al.*,[10] and analogous procedures were used to prepare methyl D-cysteinate hydrochloride, mp = 140–141° (lit. mp of L-enantiomer = 141–142°)[11] and n-butyl D-cysteinate, mp = 89–90° (lit. mp of L-enantiomer = 91°).[12] The n-hexyl ester hydrochloride was prepared by suspending 0.47 g of D-cysteine in 8.0 mL of n-hexanol that had been saturated with dry HCl and heating the mixture for 50 min at 95°. Concentration of the reaction mixture to 2 mL followed by addition of 6 mL of hexane and cooling resulted in formation of white crystals (549 mg), which were recrystallized twice from ethyl acetate, mp = 91–92° (Found: C, 44.70; H, 8.45; N, 5.80. $C_9H_{20}NSO_2Cl$ requires C, 44.70; H, 8.35; N, 5.79%). All of the alkyl D-cysteinates exhibited IR bands at 2490 cm^{-1} (SH) and 1755 cm^{-1} (C=O). The ^1H NMR spectra (90 MHz, dimethylsulfoxide-d_6) showed signals at δ4.3 (t, 1H) and δ3.05 (m, 2H) due to the cysteinate moiety in addition to the expected resonances for the alkyl groups.

Inhibition studies with aminopeptidase M were carried out at 25° in 70 mM Tris-HCl, pH 7.5, containing 0.2 mM dithiothreitol and 1% v:v ethanol. Leucine *p*-nitroanilide was used as substrate, and the formation of *p*-nitroaniline was monitored spectrophotometrically at 405 nm with a Beckman DU-40 spectrophotometer. Solutions of the inhibitors were freshly prepared in ethanol and serially diluted with ethanol to about 10^{-4} M inhibitor; this concentration was checked by sulfhydryl determination[13] both before and after each series of experiments. It was found that these solutions were stable toward oxidation for several hours, whereas aqueous solutions were not. The inhibitor was added to each assay mixture just before initiation of the reaction by addition of enzyme. The absence of upward curvature in plots of A_{405} vs time indicate that no significant oxidation of the inhibitor occurred during the initial rate measurements when 0.2 mM dithiothreitol was present.

Inhibition studies with cytosolic leucine aminopeptidase were carried out at both pH 7.5 and pH 8.4 in 50 mM Tris-HCl using the methods described above. Prior to use the enzyme was activated by incubation with 1 mM $MnCl_2$ in 20 mM Tris-HCl, pH 8.4, for 2 h at 37°.

RESULTS AND DISCUSSION

Figure 1 indicates that ethyl D-cysteinate is a very good competitive inhibitor of aminopeptidase M. The K_i value determined from the slopes of the lines in Figure 1 is 3.5×10^{-7} M. Comparison of this value with the published K_i for 2-aminoethanethiol (Table I) indicates that the carboethoxy group makes a favorable contribution of about one order of magnitude. D-Cysteine inhibits less effectively (Table I)

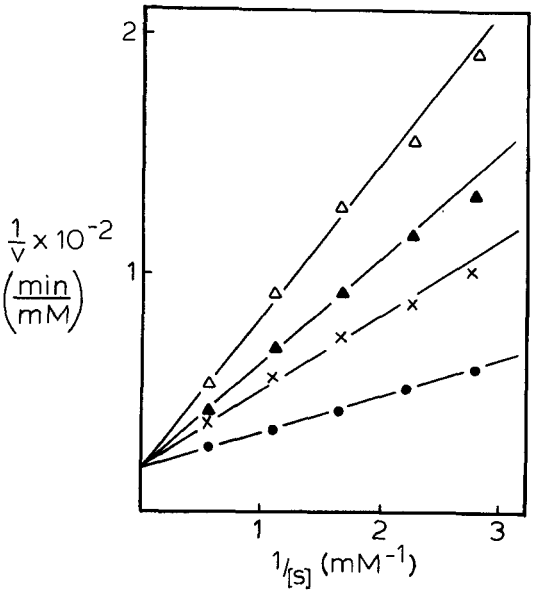

FIGURE 1 Inhibition of aminopeptidase M by ethyl D-cysteinate at pH 7.5. Rates were measured with leucine *p*-nitroanilide as substrate as described under Materials and Methods. The concentrations of ethyl D-cysteinate were (●) 0 μM, (X) 0.4 μM, (▲) 0.8 μM, and (△) 1.2 μM. Slopes and intercepts were determined by weighted least squares.[20]

TABLE I
Inhibition of Aminopeptidase M by Alkyl D-Cysteinates and Related Compounds at pH 7.5.

Inhibitor	K_i(M)
2-aminoethanethiol[a]	3.2×10^{-6}
D-cysteine[b]	4.8×10^{-5}
methyl D-cysteinate[c]	6.9×10^{-7}
ethyl D-cysteinate[c,d]	3.5×10^{-7}
n-butyl D-cysteinate[c]	1.8×10^{-7}
n-hexyl D-cysteinate[c]	3.6×10^{-7}
ethyl L-cysteinate[b]	9.6×10^{-5}

[a] Reference 7.
[b] K_i determined from Dixon plot.[21]
[c] K_i determined from double reciprocal plot.
[d] K_i from Dixon plot was 3.9×10^{-7} M.

than 2-aminoethanethiol, presumably because the anionic carboxylate group interacts unfavorably with the binding site for R_1.

The pH-variation of the kinetic parameters of aminopeptidase M suggests that the enzyme binds substrates in the amine-protonated form.[14] If the sulfur atom in our inhibitors binds to Zn^{+2}, it probably binds as a thiolate anion. Therefore, the potency of any β-mercaptoamine inhibitor should be proportional to the concentration of the zwitterionic form, $^{+}H_3NCHXCH_2S^{-}$. At pH 7.5, 15% of ethyl cysteinate exists in this form compared with 12% of aminoethanethiol and 7% of cysteine (calculated from the microscopic pK_a values in reference 15). These differences are too small to account for the differences in the K_i values of these compounds. A reasonable explanation for the relative K_i values is that the carboethoxy group of ethyl D-cysteinate interacts favorably with the binding site for R_1 and that the carboxylate group of cysteine interacts unfavorably.

As shown in Table I, ethyl L-cysteinate inhibits much less effectively than the D enantiomer. This result is consistent with our working model for the inhibition and with Chan's finding[7] that D-leucinethiol inhibits relatively weakly ($K_i = 3.6 \times 10^{-5}$ M).

Table I also presents the results of our initial studies on the effects of varying R_1. In the n-alkyl series, activity passes through an optimum near n-butyl, but the optimum is not particularly sharp. The most active compound, the n-butyl ester, is still less effective than leucinethiol by about a factor of 3, but the fact that relatively large alkyl groups can be accommodated raises the possibility that further improvements can be made by the introduction of branched or possibly cyclic groups.

Aminopeptidase M is relatively nonspecific with respect to the amino-terminal residue. K_m values show a modest preference for hydrophobic residues,[9] but k_{cat} is maximum for alanine derivatives,[9] and the variation of k_{cat}/K_m is not easily interpretable. Chan's results and our own provide evidence for a relatively hydrophobic binding site for R_1, and our results indicate that this binding site is capable of accommodating relatively large groups.

We have also tested ethyl D-cysteinate as an inhibitor of cytosolic leucine aminopeptidase (cytosolic LAP, EC 3.4.11.1), a Zn^{+2}-containing enzyme that also requires Mg^{+2} or Mn^{+2} for activity.[16] This enzyme has a stronger preference[1] for hydrophobic amino-terminal residues than aminopeptidase M. Surprisingly, no significant inhibition was observed at ethyl D-cysteinate concentrations as high as 1 mM. The reason for this result is uncertain, but it should be noted that leucinethiol also inhibits cytosolic LAP relatively weakly ($K_i = 2 \times 10^{-4}$ M).[17]

A highly specific inhibitor of aminopeptidase M would be helpful in clarifying the physiological functions of closely related enzymes in brain and other tissues.[18] Studies on the functions of aminopeptidases commonly make use of the antibiotic bestatin,[5,18] but bestatin inhibits both aminopeptidase M ($K_i = 4.1 \times 10^{-6}$ M) and cytosolic LAP ($K_i = 1.9 \times 10^{-8}$ M).[19] The alkyl D-cysteinates described here are more effective than bestatin at inhibiting aminopeptidase M and do not inhibit cytosolic LAP. As inhibitors of aminopeptidase M, the alkyl D-cysteinates are only slightly less effective than leucinethiol, and they are easier to synthesize and do not rapidly oxidize to the disulfide as does leucinethiol.[6,7,17]

In conclusion, our results indicate that esters of D-cysteine are a promising class of inhibitors of aminopeptidase M. Whether the strategy employed here can be extended to other metal-dependent aminopeptidases will need to be tested on a case-by-case basis, but the ease with which the inhibitors can be synthesized makes this testing a relatively simple task.

Acknowledgements

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