The behaviour of leucine aminopeptidase towards thiono-peptides

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Thionoleucine *S*-anilide (Leut-anilide), Leut-Gly-OEt and Leut-Phe-OMe were synthesized and shown to be competitive inhibitors of leucine aminopeptidase from pig kidney. The kinetics of inhibition were determined in the presence of leucine 4-methylcoumarin-7-amide as substrate. Although the compounds showed only moderate inhibitory potency, it was found that all were resistant to hydrolysis by the enzyme, in contrast with the reported behaviour of some thiono-peptide analogues of substrates for other Zn^{2+} -peptidases such as carboxypeptidase A and angiotensin-converting enzyme.

INTRODUCTION

The activation of leucine aminopeptidase (LAP) (EC 3.4.11.1) by certain metal ions, first reported by Johnson *et al.* (1936), has been the subject of a number of investigations (Berger & Johnson, 1939; Smith & Bergmann, 1941, 1944). The fact that Zn^{2+} could be replaced by Cd^{2+} to give an inactive enzyme that could in turn be re-activated by dialysis against Zn^{2+} strongly suggested that Zn^{2+} is an essential component of the enzyme (Himmelhoch, 1969). Inhibition by metal-ion-chelating agents has played an important role in the recognition of Zn^{2+} as a constituent of LAP and a number of other metalloproteinases.

In recent years considerable interest has been shown in the development of specific inhibitors of metalloproteinases (Petrillo & Ondetti, 1982; Vallee & Goldes, 1984; Patchett & Cordes, 1985), and these have often been designed to chelate the metal ion. The precise catalytic mechanism of metalloproteinases is not well understood, although intensive study of thermolysin and carboxypeptidase A (Vallee & Goldes, 1984) has led to suggested mechanisms for these from which extrapolations have been made to explain the behaviour of other metalloproteinases (Patchett & Cordes, 1985). One suggestion is that the Zn^{2+} of these enzymes binds the carbonyl O atom of the scissile bond of the substrate, facilitating nucleophilic attack by H_2O at the carbonyl C atom (Vallee & Goldes, 1984). The design of some potent inhibitors of angiotensin-converting enzyme has been based upon this hypothesis (Patchett *et al.*, 1980; Petrillo & Ondetti, 1982; Okuda & Arakawa, 1985; Patchett & Cordes, 1985). These inhibitors contain either a thiol or a carboxy group that is believed to chelate the metal ion on binding at the active site.

Replacement of O by S at the scissile bond of peptide substrates represents a minimal change in structure (La Cour *et al.*, 1983; Guthrie *et al.*, 1986) that might nevertheless enhance binding to the metal ion of metalloproteinases, providing a possible basis for new inhibitors of these enzymes. Recently it was shown that the thiono-peptide *N*-(furylacryloyl)-Phet-Gly-Pro is a

good substrate for angiotensin-converting enzyme, whereas the analogue in which Ala replaces Gly is not hydrolysed at all (Maziak *et al.*, 1986). Similarly, Z-Glyt-Phe was shown to be a weak competitive inhibitor of carboxypeptidase A (Bartlett *et al.*, 1982), and is also a substrate (Mock *et al.*, 1981; Bartlett *et al.*, 1982). Work by Bond *et al.* (1986) also shows that carboxypeptidase A can hydrolyse thiono-peptides. Other examples of the inhibition of Zn^{2+} -enzymes by thiono compounds include the report by Lee *et al.* (1973) that thiosemicarbazones of pyridine aldehydes are strong chelators of the metal ion in alkaline phosphatase and a study of the inhibition of thermolysin by Schiff-base derivatives of *N* α -thiocarbazoyl-L-phenylalanine methyl ester (S. Bates, D. J. S. Guthrie & D. T. Elmore, unpublished work).

Inhibitors of LAP include the naturally occurring bestatin and amastatin (Umezawa *et al.*, 1976; Aoyagi *et al.*, 1978) and hydroxamates of amino acids (Chan *et al.*, 1982). The enzyme is also strongly inhibited in competitive manner by 2-amino-1-mercapto-4-methylpentane (leucinethiol), probably as a result of interaction between the thiol group of the inhibitor and the Zn^{2+} of the enzyme (Chan, 1983). We have recently undertaken a study of sulphur-containing inhibitors of LAP and now report on the behaviour of the enzyme towards three thionoleucine compounds, Leut-anilide, Leut-Phe-OMe and Leut-Gly-OEt (I–III, X = S).

Abbreviations used: LAP, leucine aminopeptidase; -Nan, *p*-nitroanilide; -Amc, 4-methylcoumarin-7-amide; abbreviations for amino acids, peptides and their protecting groups are as recommended in Biochem. J. (1984) 219, 345–373; thiono-peptides are named by adding the letter t to the three-letter code for the amino acid carrying the S atom (Jones *et al.*, 1973); all chiral amino acids are of the L configuration.

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Comparisons were made with the corresponding oxy analogues (I–III, X = O), all of which were shown to be substrates.

MATERIALS AND METHODS

N.m.r.

N.m.r. spectra were recorded on a Bruker WM 250 spectrometer operating at 250 MHz.

Materials

The cytosolic form of LAP from pig kidney (Sigma type III-CP) was obtained from Sigma Chemical Co., Poole, Dorset, U.K. It had a specific activity of 40 units/mg (1 unit liberated 1 μ mol of Leu/min from Leu-NH₂ at pH 8.5 and 25 °C).

Leu-Nan hydrochloride was synthesized by the method of Yasutake & Powers (1981). Leu-Amc hydrochloride was synthesized by the method of Kanaoka *et al.* (1977). Leu-Phe-OMe hydrochloride was prepared as described by Beyerman *et al.* (1973), m.p. 197–199 °C (literature value 198.5–199.5 °C). Leu-Gly-OEt hydrochloride was prepared by the method of Maher *et al.* (1972) and was obtained as a gum. Leu-NHPh was synthesized by coupling Boc-Leu to aniline by using the mixed-anhydride method (Weygand & Nintz, 1965). The Boc-Leu-NHPh was deprotected with ethereal HCl, yielding Leu-NHPh.HCl, m.p. 241–243 °C. The product was homogeneous on t.l.c. in methanol (R_F 0.57) or chloroform (R_F 0.06) (Found: C, 61.78; H, 8.39; N, 12.05. C₁₂H₁₉ClN₂O requires C, 61.9; H, 8.17; N, 12.05%). ¹H n.m.r., δ (p.p.m.) ([²H₆]dimethyl sulphoxide) 11.18 (1H, s, PhNH), 8.6 (3H, broad s, NH₃⁺), 7.3 (5H, m, C₆H₅), 4.15 (1H, m, C_(\alpha)H), 1.74 [1H, m, CH(CH₃)₂; 2H, m, CH₂], 0.95 (6H, m, 2CH₃).

The sulphur compounds (I–III, X = S) were prepared by thionation of the Boc-protected oxy analogues as follows. The Boc-protected leucine derivative (I, II or III, X = O) (8.8 mmol) and 2,4-bis-(4-methoxyphenyl)-1,2,2,4-dithiadiphosphetane 2,4-disulphide (1.86 g, 4.4 mmol) were heated together in benzene under reflux for 5 h. The solvent was evaporated and the product was purified by flash chromatography on a silica-gel column. It was eluted from the column with ethyl acetate/cyclohexane (1:1, v/v) and recovered by evaporation of the solvent. The *N*-protecting group was removed by stirring the product in dichloromethane/trifluoroacetic acid (1:1, v/v) (10 ml) for 1 h at room temperature. The solvent was evaporated, and the residue was dissolved in water and washed with dichloromethane. The aqueous phase was neutralized with NaHCO₃ and the product was extracted into dichloromethane, and the extract was then dried (MgSO₄) and evaporated. The residue was converted into a hydrochloride salt by treatment with a solution of conc. HCl (5 ml) in dioxan (5 ml). After evaporation of the solvent, the hydrochloride was purified by dissolving it in methanol and re-precipitating with light petroleum (b.p. 40–60 °C). Further purification of Leut-NHPh.HCl and Leut-Gly-OEt.HCl was effected by chromatography on a 45 cm $\times$ 1 cm column of Sephadex LH-20, with elution with dry methanol. *Leut-NHPh.HCl*, yield 1.99 g (53%), had m.p. 258 °C (Found: C, 55.4; H, 7.5; N, 11.1. C₁₂H₁₉ClN₂S requires

C, 55.7; H, 7.4; N, 10.8%). ¹H n.m.r., δ (p.p.m.) ([²H₆]dimethyl sulphoxide) 8.53 (3H, broad s, NH₃⁺), 7.8 (5H, m, C₆H₅), 4.69 (1H, m, C_(\alpha)H), 1.73 [1H, m, CH(CH₃)₂; 2H, m, CH₂], 0.96 (6H, m, 2CH₃). *Leut-Phe-OMe.HCl* was obtained in 40% yield and had m.p. 153–154 °C (Found: C, 55.8; H, 7.1; N, 7.8. C₁₆H₂₅ClN₂O₂S requires C, 55.7; H, 7.3; N, 8.1%). ¹H n.m.r., δ (p.p.m.) ([²H]chloroform) 9.7 (1H, broad s, CSNH), 8.4 (3H, broad s, NH₃⁺), 7.29 (5H, m, C₆H₅), 5.3 (1H, m, PhCH₂CH), 4.6 (1H, broad s, CHCS), 3.65 (3H, m, CO₂CH₃), 3.37 (2H, m, CH₂Ph), 2.2 [2H, broad s, CH₂CH(CH₃)₂], 1.78 (1H, m, CH), 0.87 (6H, m, 2CH₃). *Leut-Gly-OEt.HCl* was obtained in 42% yield and had m.p. 54 °C (Found: C, 44.5; H, 7.9; N, 10.5. C₁₀H₂₁ClN₂O₂S requires C, 44.7; H, 7.8; N, 10.4%). ¹H n.m.r., δ (p.p.m.) ([²H]chloroform) 10.4 (1H, broad s, CSNH), 8.2 (3H, broad s, NH₃⁺), 4.8 (1H, broad s, C_(\alpha)H), 4.36 (2H, AB q, CH₂CO), 4.24 (2H, q, CH₂CH₃), 1.90 (2H, t, CHCH₂CH), 1.7 (1H, m, CH), 1.3 (3H, t, CH₂CH₃), 0.98 (6H, q, 2CH₃).

Enzyme studies

Spectrophotometric studies were carried out with a Pye-Unicam SP6-450 UV/VIS spectrophotometer connected to a Phillips single-pen recorder. The enzyme as purchased contains an excess of Mg²⁺ ions. To test whether these might affect the inhibitory properties of the thionoleucine derivatives, the inhibition of LAP by Leut-NHPh was examined in the presence of these ions and also after their removal by gel filtration of the enzyme as described by Van Wart & Lin (1981). In addition, the effects of MgCl₂ (0.05, 0.5 and 5 mM) on the thiono compounds (0.1 mM) was examined by difference spectroscopy at 25 °C in the assay buffer (see below) with a Cary model 118C spectrophotometer.

All assays were performed at 25 °C in Tris/HCl buffer prepared by adjusting an approximately 5 mM solution of Tris to pH 8.5 with dilute HCl and diluting to 5 mM. Substrates (and inhibitors where appropriate) were dissolved in buffer (690 μ l) in a cuvette of 10 mm pathlength. After thermal equilibration the reaction was started by adding enzyme solution (10 μ l, 4 units).

LAP was routinely assayed by measurement at 405 nm of the *p*-nitroaniline formed from Leu-Nan (Tuppy *et al.*, 1962). This assay was not used for inhibition studies on the thiono compounds because of a chemical interaction that occurred between inhibitor and substrate, resulting in formation of several products. Leu-Amc (Saifuku *et al.*, 1978) was found to be a satisfactory alternative substrate. Coumarinamides are normally used as fluorogenic substrates (Zimmerman *et al.*, 1976), but in the present study the hydrolysis of the coumarin derivative by LAP was monitored by following the change in u.v. absorbance due to formation of 7-amino-4-methylcoumarin ($\Delta\epsilon_{360}$ 7800 M⁻¹·cm⁻¹). Assays were conducted with several concentrations of inhibitor (0.1 to 5 mM).

Hydrolysis of Leu-Phe-OMe and Leu-Gly-OEt was monitored by measuring peptide bond cleavage at 215 nm ($-\Delta\epsilon_{215}$ 270 M⁻¹·cm⁻¹). The hydrolysis of Leu-NHPh was monitored by measuring liberated aniline in a discontinuous assay as follows. At intervals of 5 min, portions (50 μ l) of the assay solution were added to a solution of 2% NaNO₂ in ice-cold 0.5 M-HCl (0.5 ml). A solution of 2-naphthol (0.4% in 1 M-KOH) (0.5 ml) was then added and the solution was kept for 15 min at 25 °C before the absorbance of the resulting phenylazo-2-

naphthol was read at 500 nm. The concentration of aniline liberated was determined from a calibration curve of A_{500} against concentration of phenylazo-2-naphthol derived from solutions of aniline in the concentration range 0.25–4.0 $\mu\text{g/ml}$. Attempts were made to study the hydrolysis by LAP of the thiono-peptides (concentrations 0.25–2 mM) by monitoring the absorbance at 262 nm, due to the thionoamide bond, for periods up to 6h. This method was used by Mock *et al.* (1981) and Bartlett *et al.* (1982) to detect the hydrolysis of Z-Glyt-Phe by carboxypeptidase A. For Leut-NHPh an additional check was made, by using the method described above to detect any liberated aniline.

Analysis of kinetic data

Initial velocities were computed by the method of Elmore *et al.* (1963). K_m values were (a) determined from double-reciprocal plots by the Lineweaver–Burk method, and were based upon rate determinations at a minimum of eight substrate concentrations in the range 0.1–5 times K_m , and (b) computed by applying the least-squares method directly to the Michaelis–Menten equation (Roberts, 1977).

RESULTS AND DISCUSSION

Difference spectroscopy showed that Mg^{2+} ions were without effect upon the u.v. spectral properties of the thionoleucine derivatives, suggesting that no interaction occurs between the metal ion and the inhibitors. The K_i for Leut-NHPh was also unaffected by the presence of Mg^{2+} .

Double-reciprocal plots of the inhibition of LAP by the thiono compounds in the presence of Leu-Amc as substrate showed that inhibition was competitive in each case. The differences in K_i values parallel the differences in K_m values for the oxy analogues (Table 1). All the thiono compounds were resistant to hydrolysis by LAP, which contrasts with the behaviour of some other metalloproteinases towards thiono-peptides. Z-Glyt-Phe, a substrate analogue for carboxypeptidase A, is slowly metabolized by that enzyme (Mock *et al.*, 1981; Bartlett *et al.*, 1982) with a K_m of 1.1 mM. Its inhibitor constant for competitive inhibition of carboxypeptidase A was found to be 1.4 mM (Bartlett *et al.*, 1982). Bond *et al.* (1986) have shown that Z-Phet-Phe, Z-Gly-Alat-Phe and Z-Glu-Phe-Phe are all hydrolysed by carboxypeptidase A, with K_m values of 0.53, 0.81 and 0.83 mM respectively. Maziak *et al.* (1986) studied the behaviour of angiotensin-converting enzyme towards two thiono-peptides, N-

furylacryloyl-Phet-Gly-Pro and N-furylacryloyl-Phet-Ala-Pro. The former was shown to be a good substrate (K_m 1.4 mM; k_{cat} 16000 s^{-1}), comparable with its oxy analogue (K_m 4.4 mM; k_{cat} 41000 s^{-1}), whereas the latter was totally resistant to hydrolysis. The related Bz-Phet-Ala-Pro was a purely competitive inhibitor (K_i 4 mM). These findings were interpreted as showing that the S atom produces some impairment of binding in the Ala compound, and also prevents attainment of a conformation essential for catalysis to occur because of a restriction to free rotation caused by overlap of the S atom and the side chain of the Ala residue. The failure of LAP to metabolize the thionoleucine derivatives cannot be explained in this way. Although compounds (I) and (III) may be subject to conformation restrictions similar to those proposed by Maziak *et al.* (1986), compound (II) (X = S) contains a Gly residue after the scissile bond, similar to the case of furylacryloyl-Phet-Gly-Pro.

All of the thiono-peptides so far tested with metalloproteinases, including those in the present study, behave either as competitive inhibitors or as substrates, which suggests that they bind to the enzymes in a manner akin to that of normal peptide substrates. It has also been shown that the thiono-peptide bond, both in the solid state (La Cour *et al.*, 1983) and in solution (Guthrie *et al.*, 1986), has the *trans* configuration. This close structural resemblance to normal peptides probably accounts for the finding that some thiono analogues of enkephalins possess biological activity at least as good as that of the natural peptides (Lajoie *et al.*, 1984). One model for the mechanism of zinc metalloproteinases involves co-ordination of the carbonyl O atom of the scissile bond to the metal ion in the Michaelis complex (Vallee & Goldes, 1984), possibly displacing a water molecule, though variants on this idea have also been proposed (for a discussion of these points see Patchett & Cordes, 1985). If, as has been suggested (Bond *et al.*, 1986), the S atom in thiono-peptide analogues similarly binds to Zn^{2+} , then it might be expected that such compounds would be good inhibitors of metalloproteinases, since the S atom in both thioureas and thioamides is a good soft nucleophile (Jannsen, 1972). This is not borne out by the K_i values for thiono compounds, when compared for example with those reported for thiol inhibitors of metalloproteinases such as the angiotensin-converting-enzyme inhibitor captopril (K_i 0.033 nM) (Okuda & Arakawa, 1985) or the LAP inhibitor leucinethiol (K_i 22 nM) (Chan, 1983).

The indifference of LAP towards the thiono-peptide bond may be exploitable in the design of analogues of biologically active peptides. The widespread distribution of LAP in mammalian tissues means that attack at the N-terminus is a common form of degradation of both exogenous and endogenous peptides. Replacement of –CONH– by –CSNH– at this position may render peptides less susceptible to breakdown *in vivo*, thus providing a simple means of extending the half-life of therapeutically useful peptides and their analogues. At the same time the relative weak inhibitory potency of thiono-peptides should ensure that they do not seriously impair the normal functions of metalloproteinases.

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Table 1. Kinetic constant for thionoleucines and their oxy analogues

Compound	K_i (mM)	K_m (mM)	$10^4 \times V_{\text{max.}}/K_m$ (min ⁻¹)
I X = O X = S	0.19	1.1	4.5
II X = O X = S	0.4	2.04	13.48
III X = O X = S	1.97	2.9	8.6

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